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SWETS EN ZEITLINGER
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ — AMSTERDAM

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## Rectification.

This table should be substituted for table 6 on p. 215 of vol. **23**, No. 2, 1957.

### TABLE 6.

*Salmonella* species found in man and animal in the Netherlands Antilles, especially Aruba.

| | | No. of cases in the Neth. Antilles 1933—1953 | | No. of cases in Aruba 1954-1955 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| group | *Salmonella* species | man | animal | beef | goat | sheep | pig | dog | cat | canary | mouse |
| B | *S. kisangani* | | | 1 | | | | | | | |
| | *S. arechavaleta* | | | 2 | | | 1 | | 1 | | |
| | *S. saint paul* | 7 | | 4 | | | 3 | | | | |
| | *S. reading* | | | 1 | | | | | | | |
| | *S. derby* | 3 | | | | | | | 1 | | |
| | *S. typhi murium* | 19 | 3 (beef) | 31 | | | 1 | 1 | 4 | 4 | 1 |
| C. 1 | *S. oslo* | | | 8 | | | | | | | |
| | *S. denver* | | | 1 | | | | | | | |
| | *S. braenderup* | | | | | | | | | | |
| | *S. montevideo* | 16 | | | | | | | | | |
| | *S. oranienburg* | 20 | | 5 | 2 | 1 | 4 | 2 | 2 | | |
| C. 2 | *S. muenchen* | 5 | | | | | | | | | |
| | *S. manhattan* | | | | | | 3 | 1 | | | 1 |
| | *S. newport* | 16 | | 8 | | | 1 | | | | |
| | *S. glostrup* | | | 1 | 22 | | 1 | 1 | | | |
| D | *S. eastbourne* | | | 5 | | | | | | | |
| | *S. enteritidis* | 27 | | 1 | | | | | | | |
| | *S. panama* | 5 | | 18 | | | 1 | 1 | | | |
| E | *S. muenster* | 2 | | | | 1 | | | | | |
| | *S. anatum* | 2 | | 63 | 2 | | 4 | 7 | 1 | | |
| | *S. give* | 3 | | 4 | | | 2 | | | | |
| | *S. canoga* | | | | | 1 | 1 | | | | |
| F | *S. abaetetuba* | | | | | | 2 | | | | |
| G | *S. havana* | | | 45 | | | 6 | | 1 | | |
| H | *S. carrau* | 1 | | | 4 | | 2 | | | | |
| | *S. thompson* | 1 | | | | | | | | | |
| | *S. typhi* | 55 | | | | | | | | | |
| | *S. bredeney* | 3 | | | | | | | | | |
| | *S. illinois* | 1 | | | | | | | | | |
| | *S. paratyphi B* | 29 | | | | | | | | | |
| | *S. cholerae suis* | 1 | 2 (pig) | | | | | | | | |
| | *S. rubislaw* | 1 | | | | | | | | | |

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# SOME UNSOLVED PROBLEMS OF YEAST TAXONOMY

by

**J. A. BARNETT**

(Received November 24, 1956).

> "Taxonomy is built upon the basic fields of morphology, physiology, ecology and genetics. Like other scientific disciplines it is a synthesis of many kinds of knowledge, theory, and method, applied in this case to the particular field of classification. Its potentialities and its limitations are largely those of the basic fields whose raw materials it utilises."
>
> (Mayr, Linsley and Usinger, 1953).

If we accept Mayr's view, and I think we must, we can expect any system of classification to change with advancing knowledge, at least to some extent. The Dutch school of Lodder and her co-workers has given us an integrated classification of the yeasts (Lodder and Kreger-van Rij, 1952); and it is partly my purpose here to consider some developments which may alter their system, or alternative systems, such as that of Kudriavzev (1954).

Guilliermond (1920) defined yeasts as "unicellular fungi of biochemical interest, spherical or oval in shape, and which multiply by budding" (my italics); and, pragmatically, the important thing about yeasts is their biochemical behaviour.

Indeed, since morphological differences are often small, biochemical characterisation is a major feature of their present-day classification (see Table 1); and it is this aspect of yeast taxonomy with which I am particularly concerned.

There are two kinds of improvement we might hope for:

TABLE 1.

Results of biochemical tests used in characterising 12 *Saccharomyces* species.

| | | *S. acidifaciens* | *S. bailii* | *S. bisporus* | *S. delbrueckii* | *S. fermentati* | *S. florentinus* | *S. lactis* | *S. marxianus* | *S. mellis* | *S. pastori* | *S. rosei* | *S. rouxii* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | A | + | + | + | + | + | + | + | + | + | + | + | + |
| | F | + | + | + | + | + | + | + | + | + | + | + | + |
| Galactose | A | ± | — | + | + | — | + | + | + | ± | — | — | + |
| | F | — | — | — | + | — | + | + | + | — | — | — | — |
| Sucrose | A | — | — | — | — | + | + | + | + | — | — | + | — |
| | F | — | — | — | — | + | + | + | + | — | — | + | — |
| Maltose | A | — | — | — | — | + | + | + | — | — | — | — | + |
| | F | — | — | — | — | + | + | — | — | — | — | — | + |
| Lactose | A | — | — | — | — | — | — | + | ± | — | — | — | — |
| | F | — | — | — | — | — | — | + | — | — | — | — | — |
| Raffinose | | | | | | $+\frac{1}{3}$ | $+\frac{1}{1}$ | $+\frac{1}{3}$ | $+\frac{1}{3}$ | | | $+\frac{1}{3}$ | |
| Inulin | | | | | | | | | + | | | + | |
| Ethanol | | ± | — | — | — | ± | — | + | + | — | — | ± | ± |
| Arbutin split | | — | — | — | — | — | — | + | + | — | — | — | — |
| $KNO_3$ | | — | — | — | — | — | — | — | — | — | — | — | — |

A: assimilation; F: fermentation. Data from Lodder and Kreger-van Rij (1952).

A. Those which make it easier or surer to sort out different strains of yeast into defined genera and species;

B. Those which give more precise information about the characteristics of each species.

Although these two categories overlap, a statement of them emphasises the distinction between developing a key for identification on the one hand, and providing a thorough-going description of the important differences between the species on the other. Yeast taxonomists are concerned both with sorting strains into species and also characterising the species themselves.

Consideration of these two possible kinds of improvement has led me to recognise four major unsolved problems of yeast taxonomy.

The first two are concerned mainly with identification.

1. There is a need to devise different sets of biochemical tests for different groups of yeasts.

2. It would be helpful to have a quantitative evaluation of how

many tests are necessary to separate different strains into already named groups, say species. (This is the problem which is least likely to be solved and will be considered last.)

The other two problems are chiefly concerned with characterising the species.

3. Each biochemical activity described should be considered quantitatively.

4. The conditions for each of these tests need to be assessed critically.

## DIFFERENT TESTS FOR DIFFERENT GROUPS.

The main biochemical technique used in early work on yeast classification was a study of the fermentation reactions (*e.g.* STELLING-DEKKER, 1931). Many wild yeasts, however, have little or no ability to ferment sugars. For instance, all *Rhodotoruloideae*, most *Trichosporoideae*, and a large number of the genus *Candida* are said to be incapable of fermenting any sugars (LODDER and KREGER-VAN RIJ, 1952). Yeasts like these may be of considerable practical importance. We are, ourselves, concerned with a group of yeasts, isolated from foods containing much sugar, such as honey and concentrated fruit juices, which appear to be capable of fermenting only glucose (KLUYVER, 1931), and, it follows, fructose and mannose (INGRAM, 1950). *Saccharomyces mellis* is an example (LODDER and KREGER-VAN RIJ, 1952).

For classifying non-fermenting yeasts, LODDER (1934) introduced growth tests with 5 sugars. This was in fact a recognition of my first point: that once we get past the stage where morphology is useful, it is often helpful to use different sets of tests for distinct groups of yeasts. Other carbon compounds than LODDER's sugars (see Table 1) may be used. Examples are the pentoses, xylose and arabinose; the disaccharides, trehalose and cellobiose; and fumaric and citric amongst the organic acids. WICKERHAM (1951) has found such compounds useful for his studies of the genus *Hansenula*; KUDRIAVZEV has used them in his classification of the sporing yeasts, and it is possible that we too may find them helpful in elucidating osmophilic *Saccharomyces* (BARNETT and INGRAM, 1955).

On the other hand, with few exceptions, LODDER and KREGER-VAN RIJ (1952) have used the same 5 sugars for both growth and fermentation tests for all species. It is important to point out that these are not 10 independent tests (e.g. compare sucrose assimilation and fermentation in Table 1). This must have been appreciated by workers in the Dutch school, since they held that disaccharides are hydrolysed before they can be fermented or oxidatively assimilated (LODDER, 1934; KLUYVER and CUSTERS, 1940): thus, for instance, the presence or absence of one particular sucrase may be the deciding factor as to whether a yeast both ferments and assimilates sucrose; so two tests may be employed to detect one enzyme.

In devising a time-table for identification procedure, WILES (1955) made the point that the "sugar fermentations other than glucose" can be postponed

> so that the results for this sugar can be observed. If this sugar is not fermented in three days, it is most unlikely that any other sugars will be fermented..

This agreed with KLUYVER (1931) who stated that if a yeast ferments any sugar, it will ferment glucose. We could go further than WILES and say that it is unnecessary to carry out fermentation tests on sugars which cannot be used aerobically, because a sugar not assimilated oxidatively is not fermented (cf. HUGH and LEIFSON, 1953).

Thus LODDER and KREGER-VAN RIJ (1952) do not really have 10 effective tests for their yeasts: if only glucose is assimilated, for instance, then all the fermentation tests, save the glucose one, give no more information. However, when they discuss the number of compounds which can be used, they say that

> an unlimited and irrational application of arbitrary compounds in assimilation tests may well lead to an undesirable splitting in dwarf species.

But until we know a great deal more about yeast comparative biochemistry, any selection is bound to be arbitrary, faute de mieux.

SCHULTZ and his colleagues advocated nitrogen and sulphur assimilation tests, using amino acids as the sole N- and S- sources (SCHULTZ and POMPER, 1948; SCHULTZ and MCMANUS, 1950). BEECH (1956) has suggested a new approach, examining chromatographically amino acids produced in metabolism; and further possibilities are indicated by the work of SPENCER and SALLANS (1956) who investigated the production of polyhydric alcohols by osmophilic yeasts.

So we come to my second problem which is to consider critically how to estimate the sorting capacity of a group of tests. But we shall discuss this question last and consider now the problem of using biochemical tests which are quantitative.

## QUANTITATIVE TESTS.

SIMPSON (1945) emphasised that

> "Variation within a group at one time is as much a character of the group as is any so-called constant characterization and may be more important and more characteristic than any demonstrable constancy... it cannot be too strongly emphasised that some estimate of variation is inherent in any valid classification."

Following SIMPSON, we can say that classification is not concerned with individual strains, but only with groups of strains. In fact morphologists do not hesitate to use ranges of sizes, even overlapping ranges, as taxonomic criteria. For example, LODDER and KREGER-VAN RIJ (1952) differentiate between *Rhodotorula rubra* and *Rhodotorula mucilaginosa*, because in the former the cells are "long-oval" and in the latter they are "round to oval". It seems reasonable to expect that classification in terms of magnitude of biochemical activities might be useful too.

Thus my third point is that to write + or — for a fermentation or growth reaction is inadequate. PIRIE (1955) says

> It would be.......... better if we got rates of production...., even if it were only an indication of the order, e.g. 1, 100, 10,000 or 1,000,000 molecules per cell per second. This would prevent similar weight being given to the presence of widely different amounts of enzyme.

This view contrasts with that of LODDER and KREGER-VAN RIJ (1952). They used Einhorn tubes for fermentation tests, although they say that Durham tubes are more sensitive:

> in some cases in which the fermentation is very weak or particularly slow we.... occasionally found a positive result in a Durham tube when the Einhorn tube yielded a negative outcome.

Further, they say that with an Einhorn tube it may be impossible to detect fermentation which is obvious immediately with a Warburg manometer.

We have made some attempt to follow PIRIE's suggestion, in calculating β-glucosidase activity in yeasts in terms of molecules split per cell per second (Barnett, Ingram and Swain, 1956). However, it would probably have been better to calculate the activity in terms of dry weight or Kjeldahl nitrogen, because budding and a tendency to flocculate make yeast cells difficult to count, quite apart from errors which may be expected anyway from haemacytometer counts (Biggs and Macmillan, 1948).

The use of this simple + or — distinction can be misleading in two ways. First the minus sign may mean, simply, low activity, and therefore give a false impression that there is none: this point is in fact made by Lodder (1934) with respect to fermentation tests. Secondly, consider a breakdown which can be measured along a scale from 0 to 100%, where every observation from 0 to 50 is recorded as —; those > 50 as + : then 2 strains, described as + and —, may not differ significantly. Strain (1) giving a reading of 50 may be differentiated from strain (2) at 51, but not from strain (3) at zero. This criticism applies wherever the line between + and — is drawn. It is necessary to use a minimum of three categories, such as ++, + and — to give any useful information: in this case, there can be a known minimum distinction between ++ and —.

We have found that this kind of criticism applies to tests involving splitting of the phenolic β-glucosides aesculin, arbutin and salicin

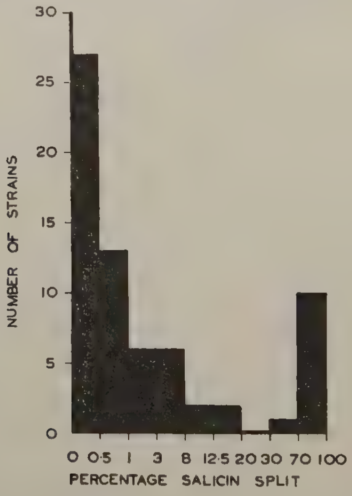

Fig. 1. Amount of salicin split by 65 yeast strains. Cell-suspensions grown on malt-yeast-glucose-peptone-agar and shaken 20 hr. with 0.25% salicin as only C-source in synthetic medium (see Barnett *et al.*, 1956).

(Barnett and Swain, 1956; Barnett, Ingram and Swain, 1956). Fig. 1 shows the scatter of 65 salicin-splitting measurements taken under standard conditions described in the last mentioned paper. For these kinds of data, arbitrary categories of + and — alone are almost useless.

## BIOCHEMICAL CONDITIONS FOR TESTS.

Our work on the splitting of $\beta$-glucosides by yeasts led us to my further problem: that of studying the conditions for each test, such as the nutritional state of the yeast. We have found that a medium on which good growth can occur may be inadequate for the development of $\beta$-glucosidase activity. (Table 2; Barnett and Swain, 1956; Barnett *et al.*, 1956). The difference in performance of the yeasts may be due to the fact that in the synthetic medium ammonia is the sole nitrogen source, while in the complex one, there are amino acids and other peptone constituents.

TABLE 2.

Response to 3 $\beta$-glucosides of 6 yeast strains when grown on 2 alternative media.

| | Synthetic growth medium | | | Complex growth medium | | |
|---|---|---|---|---|---|---|
| | aesculin | arbutin | salicin | aesculin | arbutin | salicin |
| *Kloeckera antillarum* NCYC 37 | ++ | — | ++ | ++ | + | ++ |
| *Pichia membranaefaciens* NCYC 54 | — | — | — | ++ | + | + |
| *Saccharomyces acidifaciens* CBS 685 | — | — | — | ++ | — | + |
| *Saccharomyces bisporus* CBS 702 | — | — | — | ++ | — | + |
| *Saccharomyces cerevisiae* NCYC 231 | — | — | — | ++ | + | + |
| *Sacch. rouxii* var. *polymorphus* CBS 713 | + | — | — | ++ | + | + |

Data from Barnett *et al.* (1956). N.B. all these species are given as arbutin-negative by Lodder and Kreger-van Rij (1952). (NCYC: National Collection of Yeast Cultures; CBS: Centraalbureau voor Schimmelcultures).

This observation of ours is comparable with that which led Lodder and Kreger-van Rij (1952) to abandon the nitrogen assimilation tests with $(NH_4)_2SO_4$, asparagine and urea, after Wickerham (1946) had shown that all the strains he tested used these compounds if sufficient B-complex vitamins were present. Thus, using Lodder (1934) as a guide we might have inferred, incorrectly, that *Kloeckera* species are totally incapable of growing with ammonium sulphate as the sole source of nitrogen. No one has yet followed up Wickerham's work by publishing a system of nitrogen assimilation reactions for standard conditions of vitamin deficiency!

There are two alternative views which could be taken by a taxonomist of the kind of observation made by Wickerham and ourselves. On the one hand, he could say that it is a *sine qua non* for taxonomy that a useful character is one which helps to predict others accurately; and there is no *a priori* reason why such a character should not be one which occurs under standard conditions of nutritional deficiency. Or, on the other hand, it could be held (as apparently do Lodder and Kreger-van Rij) that as taxonomy is just a useful card index for practical people, it should be concerned with the overall potentialities of the yeast, rather than with its behaviour in some special circumstances. If this last view is thought to be better, and I think it is, it may be necessary to re-examine the conventional tests to see if they provide something like maximal conditions for enzyme production, as well as if the tests themselves are sensitive enough. Unfortunately, what may be a maximal medium for the production of one enzyme may not be so for another, even for the same strain; so it may be important to try to avoid characters which tend to be excessively labile (cf. Cowan, 1955).

### Number of tests used.

Perhaps we can now consider our most difficult problem of how many dichotomy (i.e. + or —) tests are necessary to separate different strains into already named groups, say species? At first we might imagine that it would be ideal to have a separate diagnostic test for each species, but this is in fact not the case. Barnett and Ingram (1955) suggested that it would seem best to use test-substrates which give 50% positive and 50% negative responses, making it possible, for instance, to distinguish between 4 species with only 2 tests. Thus, in this table, each species has a response pattern distinct from that of each of the other 3 species.

| 4 SPECIES | | A | B | C | D |
|---|---|---|---|---|---|
| 2 TESTS | a | + | + | — | — |
| | b | — | + | + | — |

In this way, the maximum possible number of species which can be separated by n tests is $2^n$; or, if there are k species, then the minimum number of tests for sorting is $\log_2 k$. But, if any of the following 4 assumptions are untrue, the number of species which can be separated by n tests will be $< 2^n$; or, putting it the other way, the minimum number of tests necessary for sorting will be $> \log_2 k$.

(1) The answers + or — correspond to some qualitative difference which is not, for instance, just a difference of degree, masked by an insensitive test. (Otherwise, there may be borderline cases awkward to classify: see previous discussion.)
(2) The tests a, b, c,.... etc. must each separate the species A, B, C,.... etc. into equal groups: half the species must be positive for each test, and half negative.
(3) The tests must be independent; the results of any one test being in no way related with those of another.
(4) The number of species under consideration must be a power of 2.

In practice, however, (2) (3) and (4) practically never hold. Nor, probably, does (1); but in the following discussion, assumption (1) is made: *i.e.* that + and — correspond with reality.

In the following argument, cases in which the results of a test are either all + or all — are excluded, and so is any test which gives results identical or reciprocal with those of another: thus we would exclude both glucose tests, sucrose fermentation and $KNO_3$ assimilation in Table 1. It has been seen that n tests can distinguish completely between $2^n$ species at most. Conversely, if there are k species, at least $\delta + \{\log_2 k\}$ tests are required to distinguish between them: where $\{\log_2 k\}$ = the integral part of $\log_2 k$, and $\delta = 0$ if $\log_2 k$ is an integral value, $= 1$ if $\log_2 k$ is a non-integer.
Thus we can write out the following table:

| No. OF SPECIES | 2 | 3, 4 | 5–8 | 9–16 | 17–32 | 33–64 | 65–128 | 129–256 |
|---|---|---|---|---|---|---|---|---|
| MINIM. OF TESTS | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

Further, with the above tenets (*i.e.* excluding tests which give no information), it can be proved that the maximum number of tests necessary is k — 1[1]). The worst possible case is the arrangement in which each species gives a positive response to one test only. So, for any practical situation, the number of tests necessary for sorting strains into k species lies between $\delta + \{\log_2 k\}$ and k — 1. It is, however, not very helpful to the taxonomist with 256 species to be told to use some number of tests between 8 and 255. On the other hand, it is possible, as BARNETT and INGRAM (1955) suggested, to keep down the number of tests by using those which tend to be independent and approach 1 : 1 sorting for the species under study.

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[1]) As in a (k — 1) – dimensional vector space, any k vectors are linearly dependent (see BIRKHOFF and MACLANE, 1948).

By using more than 2 categories, such as ++, +, —, if these responses are repeatable for each strain, fewer tests are necessary. For tests giving 3 such categories, the minimum number of tests needed is $\delta + \{\log_3 k\}$, for 4 categories $\delta + \{\log_4 k\}$, and so on. So we can extend the table we wrote out before, to cover tests which are not just + and —, but for different numbers of categories, T, with different numbers of species, k. The entries in this table represent the minimum number of tests required in each case $\delta + \{\log_T k\}$.

| | | k | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 5 | 9 | 17 | 33 | 65 | 129 |
| T | 2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| | 3 | 1 | 2 | 2 | 3 | 3 | 4 | 4 | 5 |
| | 4 | 1 | 1 | 2 | 2 | 3 | 3 | 4 | 4 |
| | 5 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 4 |
| | 10 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 3 |

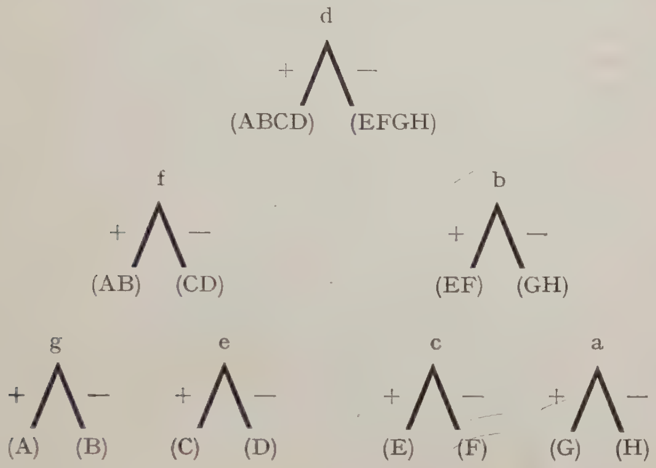
In this way, it is obvious that we need fewer tests if they are repeatable and quantitative.

Furthermore, if we follow WILES (1955) and have an identification procedure, like those for identifying cations in school chemistry books, where we wait for a given result to tell us which of two tests we should use next, we minimise the number of tests necessary for any one identification, though our whole battery may be very inefficient. For example, consider 8 species (A to H) and an inefficient combination of tests, (a to g), k — 1 tests being necessary.

| SPECIES | | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|---|
| TESTS | a | + | + | + | + | + | + | + | — |
| | b | + | + | + | + | + | + | — | — |
| | c | + | + | + | + | + | — | — | — |
| | d | + | + | + | + | — | — | — | — |
| | e | + | + | + | — | — | — | — | — |
| | f | + | + | — | — | — | — | — | — |
| | g | + | — | — | — | — | — | — | — |

In this case it is possible to identify any one of the species using

3 tests only. Thus, test d separates the species into 2 equal groups, the + species being then separated by test f and the — species by test b; and so on:



For identifying A or B, only tests d, f and g are required; for C or D only d, f and e; and so on. This procedure is comparable with using one set of tests for one species group and different tests for another.

The kind of sorting problem which crops up in practice can be illustrated with reference to Table 1. The *Saccharomyces* species characterised there in the main cover the former genus *Zygosaccharomyces*. We are particularly interested in identifying such organisms; and because they are a fairly uniform group, it is from biochemical tests that we hope to get our best differentiation. However, using just the tests of LODDER and KREGER-VAN RIJ (1952) given in Table 1, and accepting their results, it may not be possible to differentiate within the two groups (I) *S. acidifaciens*, *S. bisporus* and *S. mellis*; and (II) *S. acidifaciens*, *S. bailii*, *S. mellis* and *S. pastori*. (The nomenclature throughout this paper is that of LODDER and KREGER-VAN RIJ, 1952).

## CONCLUSION.

In discussing each of these problems, I have tried to convey the idea that we should have closer control over and understanding of our taxonomic procedures. The first problem, that of devising specific tests appropriate to each group of yeasts, implies that we should try to find out more about the metabolic patterns of each kind of yeast. The second problem suggests the need for a theory which would tell us, with some degree of precision, how effective our tests are. The third and fourth points are concerned with obtaining precise information about the biochemistry of the tests themselves.

In the last context, I would like to suggest further that wherever possible we ought to try to study unit processes (cf. CLARKE, 1955). To take an example, growth on aesculin as sole carbon-source has been used as a biochemical test (WILES, 1955). An enzyme from the yeast splits the aesculin into the phenolic aglycone, aesculetin, and glucose which is then used for growth. Some yeasts may, however, be unable to grow appreciably in the presence of the aesculetin; and so, with this technique, we may be lumping together those strains which cannot split aesculin under the conditions of the test, and those which are particularly sensitive to aesculetin (BARNETT *et al.* 1956). Measurement of the actual splitting of aesculin, however, is less likely to involve as many complications as measuring growth on it. Basing classification as nearly as possible on single enzyme processes will probably mean that there is for the future less likelihood of the need for early revision of the system. And it will have the further advantage of integrating the identification key with the description of the organisms. KUDRIAVZEV (1954) thinks it important to distinguish between activities due to constitutive and adaptive enzymes.

A critical approach of this sort may also have application in genetics (cf. HALDANE, 1954) where taxonomic methods are sometimes used (ROBERTS, 1950). In elucidating the genetic basis for the presence or absence of, say, a fermentation reaction, it is important to know the actual reason for a negative reaction: whether it is the insensitivity of the test, the need for a vitamin, a difference in cell-wall permeability, or the lack of hydrolase or phosphorylase. There are many such opportunities for genetic differences to be masked by phenocopies in such "presence-or-absence" characters.

Agreeing with HUXLEY's (1940) sentiment that "To hope for the new systematics is to imply no disrespect for the old"; my intention has been simply to anticipate changes which may occur with advancing knowledge. It is best to think about such changes as much as possible before they occur, rather than after when we may be saddled with a new classification with too many snags. Changing a classification is very inconvenient to all its users, so the more energetically such ideas can be discussed, as far as possible in advance, the better. I am not proposing that we should indulge in name-changing activities, like the recent suggestion that the *Saccharomyces* species using lactose should be called "*Dekkeromyces*" (WICKERHAM, 1955). Even though this gesture was charming, the confusion likely to result makes it seem hardly worth-while: indeed, KUDRIAVZEV (1954) anticipated WICKERHAM by calling this group "*Fabospora*", because of the bean-shaped spores. On the other hand, a more careful description of the characteristics of a species should not lead to confusion; it should make taxonomy more useful.

## Acknowledgement.

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# A SIMPLE METHOD FOR THE DETECTION OF LIPOLYTIC ACTIVITY OF MICRO-ORGANISMS AND SOME OBSERVATIONS ON THE INFLUENCE OF THE CONTACT BETWEEN CELLS AND FATTY SUBSTRATES [1]

by

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(Received July 24, 1956).

## 1. Introduction

The detection of lipolytic activity of micro-organisms is of value in the study of spoilage of food and for taxonomical purposes.

While we were investigating esterases of micro-organisms there appeared in this journal a paper on the same subject by Bulder (1955). As this paper made clear that there is still need of a simple and reliable method to detect lipase activity in micro-organisms, we offer here a new method.

As Bulder has discussed extensively the three most important plate-methods in use up till now, we shall only present some further considerations.

In the first place it seems opportune to point out that the way, in which the method of Knaysi is described by Bulder, may be the cause of some misunderstanding. In the method of Knaysi (1941) there is no question of Nile blue being replaced by neutral red, but the cationic components of the basic dyes, which are slightly toxic, are replaced by the free bases of these dyes. These free bases are precipitated by adding a 1 N NaOH solution to the dye solution.

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The in water unsoluble free bases dissolve in fats with a colour different from the original dye. If fatty acids are liberated by lipase the original colour comes back.

Tributyrin according to the ANDERSON'S test (1934) also mentioned by BULDER, was extensively used in our experiments. Indoxyl butyrate was suggested by KAMAL MUFTIC (1954). It has the advantage that the hydrolysis of this substrate is easily observed, because of the direct formation of indigo blue. For three reasons, however, these substrates are not very suitable as a general method in judging lipolysis.

In the first place there are some bacteria with a lipase, which do not hydrolyse tributyrin or other butyric acid esters, although they do hydrolyse the true fats. Our experiments showed, that lipase can be distinguished from ali-esterase. For instance *Pseudomonas aeruginosa* has a lipase which splits high fatty acid esters, but not tributyrin and ethyl butyrate, although an ali-esterase, active on tributyrin, is also present in this micro-organism (SIERRA, 1956). Also *Micrococcus lysodeikticus* Fleming has a lipase, which fails to hydrolyse tributyrin as well as ethyl butyrate (SIERRA, 1956).

In the second place we found a converse case e.g., *Bacillus megaterium* which does not split high fatty acid esters, although it is able to attack tributyrin as well as triacetin, as was shown by manometric estimation in washed cell-suspensions (SIERRA, 1955).

In the third place the results obtained with tributyrin may be misleading, a fact which was already noted by LONG and HAMMER (1937a). It is well-known that tributyrin can hydrolyse spontaneously, so that it is necessary to introduce corrections when measuring tributyrinase activity with a Warburg apparatus. Therefore, it seems doubtful, that the strains tested by LONG and HAMMAR (1937b) could actually split tributyrin but no fats.

The method of BULDER (1955), which requires a nebuliser to spray minute droplets of olive oil on the nutrient agar plates after the inoculation, has the disadvantage that it is hardly possible to prevent infections from the air. Although the method is direct, a microscope is needed in order to observe the changes in the droplets.

Now already GOMORI (1945) used Tween 60 as substrate in histochemical studies of lipase in tissues. His method was used later on by BAYLISS and co-workers (1948) who studied the localisation of intracellular lipase in some micro-organisms. ARCHIBALD (1946) recommended Tweens in the estimation of serum lipase. In our own experiments we found that the Tweens are very suitable for measuring lipase activity of *Pseudomonas aeruginosa* (SIERRA, 1956).

It seemed therefore indicated to try Tweens for the detecting of lipolytic activity in a plate method. In the method to be reported, the lipolytic activity is easily recognised by the appearance of a crystalline precipitate in the plates, which is probably due to the insoluble calcium salt of the fatty acid liberated by lipolysis.

## 2. EXPERIMENTAL.

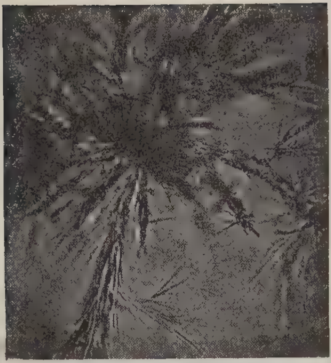
Three Tweens were tested as substrates for lipase. Tweens are water-soluble high fatty acid esters of a polyoxyalkylene derivative of sorbitan (Atlas Surface Active Agent, 1948, Atlas Powder Co., Wilmington, Delaware); they differ only in the particular fatty acid component. Tween 40 is a palmitic acid ester; Tween 60 a stearic acid ester; and Tween 80 an oleic acid ester. The Tweens are stable in diluted alkaline and mineral acid solutions. They are virtually neutral, and non-volatile as well as heat stable.

A peptone agar medium was prepared with each of the Tweens. The basal medium is composed as follows: Difco Bacto-peptone 10.0 g, NaCl 5.0 g, $CaCl_2.1H_2O$ 0.1 g, destilled water 1000 ml. The pH was adjusted to 7.4.

500 ml of the medium were sterilised in the flasks and cooled to 40—50°C. The Tweens were sterilised separately by autoclaving twenty minutes at 120°C. Then five ml of each Tween was added to the peptone agar flasks (about 1 % final concentration of Tween) and shaken until complete solution. From this clear and transparent medium (pH 7.0—7.4) plates were poured, on which a suspension of the micro-organism to be tested was streaked. The cultures were maintained by frequent transfers on peptone agar slants, a fresh slant being prepared eighteen to twenty hours before the inoculation of the plates.

The plate-cultures were incubated at 30°C. and 37°C. according to the optimal growth temperature for each micro-organism.

It was found, that if the micro-organism tested had a lipolytic activity an opaque halo could be easily observed around the colonies. When studied microscopically these opaque haloes turned out to consist of crystals of the formed calcium soaps. The various Tweens can be distinguished from each other by their characteristic crystals formed in the haloes. See Figures 1, 2 and 3.



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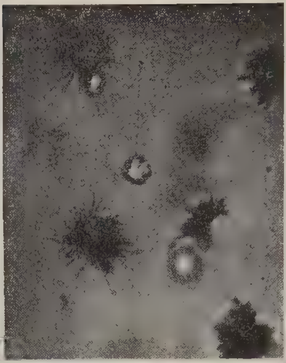

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Fig. 1. Crystals on a plate with Tween 40 (250 ×).
Fig. 2. Crystals on a plate with Tween 60 (250 ×).
Fig. 3. Crystals on a plate with Tween 80 (750 ×).
Micrographs by Mr. R. Zagt.

The fact, that the opaque zone is formed only in the immediate vicinity of the colonies, whereas the rest of the medium remains transparent, demonstrates that the appearance of the crystalline form is not due merely to any interference of the Tweens with some constituent of the medium. Moreover, plates not inoculated dry up in the incubator before any change in their appearance can be observed.

### 3. Comparison of the Eijkman, Bulder and Tween tests.

As it was observed that some species of bacteria were negative on the Eijkman (1901) plates, but positive on the Tween plates, it was decided to compare both methods with the Bulder (1955) spray test. The results obtained are shown in Table 1.

The table shows that in eleven cases there is a complete agreement between the three methods. In five cases the Eijkman test was negative whereas the Bulder and Tween tests were positive. *Mycobacterium phlei* was only positive on the Tween plates. It is noteworthy that *Micrococcus lysodeikticus* was only negative when tested on Tween 60 and on Tween 80, and *Streptococcus liquefaciens* on Tween 80.

### 4. Discussion

Against the use of Tweens instead of oils and fats may be objected that these are not glycerides as are the natural fats and oils. However, Archibald (1946) using Tweens, Nachlas and Seligman

TABLE 1.

Comparison of the lipolytic activity of some micro-organisms achieved by means of EIJKMAN (1901), BULDER (1955), and Tween test.

The figures between brackets indicate the number of days after which a positive result could be observed. The culture plates containing no lipolytic bacteria were kept in observation during a month.

| Micro-organism 1) | EIJKMAN test beef tallow | BULDER test olive oil | Tween test | | |
|---|---|---|---|---|---|
| | | | Tween 40 | Tween 60 | Tween 80 |
| *Escherichia coli* | — | — | — | — | — |
| *Pseudomonas putida* | — | — | — | — | — |
| *Bacillus megaterium* | — | — | — | — | — |
| *Pseudomonas aeruginosa* | + (3) | + (1) | + (2) | + (2) | + (2) |
| *Micrococcus pyogenes* var. *aureus* | + (3) | + (2) | + (2) | + (3) | + (3) |
| *Vibrio metschnikovii* | + (7) | + (3) | + (3) | + (3) | + (3) |
| *Bacillus subtilis* 422 | + (4) | + (3) | + (3) | + (3) | + (3) |
| *Bacillus pumilus* | + (4) | + (2) | + (1) | + (2) | + (2) |
| *Streptococcus liquefaciens* | + (5) | + (2) | + (2) | + (2) | — |
| *Micrococcus lysodeikticus* | + (7) | + (3) | + (2) | — | — |
| *Bacillus macerans* | + (2) | + (2) | + (2) | + (2) | + (2) |
| *Bacillus mycoides* | — | + (2) | + (2) | + (2) | + (2) |
| *Bacillus cereus* | — | + (3) | + (2) | + (3) | + (3) |
| *Bacillus pumilus* 33 | — | + (2) | + (3) | + (2) | + (3) |
| *Vibrio tyrosinatica* | — | + (2) | + (1) | + (1) | + (1) |
| *Bacillus subtilis* 4 | — | +(12) | + (4) | + (4) | + (4) |
| *Mycobacterium phlei* | — | — | + (2) | + (2) | + (2) |

1) The species of the sporeforming bacteria were determined with the aid of the classification proposed by SMITH and co-workers (1952).

(1949) using $\beta$-naphthol esters, RAVIN and SELIGMAN (1953) indoxyl esters, and HUGGINS and LAPIDES (1947) esters of p-nitrophenol, did show that esters, other than glycerides, of long chain fatty acids are readily hydrolysed by lipase. For this reason NACHLAS and SELIGMAN (1949) amplified the definition of "lipase". According to them "lipases" are those enzymes which hydrolyse esters of long chain fatty acids, including fats and oils.

As far as can be inferred from our own experiments this seems also to hold for bacterial lipase. *Mycobacterium phlei* seemed to be an exception, since it only hydrolyses Tweens. To make quite sure, that *Mycobacterium phlei* really cannot split olive oil, this micro-organism was tested with the titrimetric technique as described by Noyons (1951). In these experiments resting cell-suspensions of the micro-organism, taken from a nutrient broth culture, were brought into contact with an emulsion of the fatty substrate during seventeen hours (at 37°C. in a shaker). The titration values amply showed that this micro-organism can split olive oil and this was also the case with *Bacillus subtilis*. The interpretation of these experiments is reserved for a future discussion.

Usually it is assumed that the lipases are enzymes of low specificity. However, there seems to be little experimental proof for this statement, since it is difficult to isolate lipase as a pure enzyme. The fact that many natural fats and oils are hydrolysed by the same enzyme preparation or micro-organism is not an indication for its low specificity, because these substances have always a few long chain fatty acids in common. Our experiments seem to indicate that only the lipase of *Micrococcus lysodeikticus* is specific, because only Tween 40 is hydrolysed. That beef tallow and olive oil are also hydrolysed is not a contradiction because both substances contain about 25% palmitic acid. Also the case of *Streptococcus liquefaciens*, being active only on Tween 60 and 40, points out some specificity. For the study of the specificity of lipases it seems to be indicated to use Tweens.

It is clear, that the last six bacteria in Table 1 should be able to hydrolyse beef tallow, because olive oil and the three Tweens are hydrolysed. The fact that these bacteria are negative in the Eijkman test, must be ascribed to the lack of a direct contact between the cell and the fat. This question has been discussed by Bulder (1955) who compared his own method with the Eijkman test for some twenty species. He found an excellent agreement between both methods, notwithstanding there was an exception for *Sarcina flava* which apparently behaved whimsically. Our own experiments suggest that lipase formation in those species which did not show agreement in both tests, is adaptive, because on the Tweens and Bulder plates all the colonies without exception showed lipase activity. This observation is confirmed by our titrimetric results mentioned above, because in these experiments we used a resting cell suspension so that mutation of a few cells and their subsequent selection might not play a part. Under these conditions the genetically unaltered cells form lipase, or, under the influence of the fatty substrate, activate a previously inactive lipase.

The fact that *Mycobacterium phlei* did not show any lipase activity in the BULDER test, and *Bacillus subtilis* only after a long time, may indicate that there is an insufficient contact between the cells and the substrate to induce enough lipase formation for detection in the surrounding droplets. This seems actually to be the case, as these two strains prefer an olive oil emulsion (titrimetric method) or the water soluble Tweens.

It is usually assumed that the formation of adaptive enzymes occurs inside the cell. In which way a water insoluble substrate may cause a stimulation is not yet clear. It is, however, possible that olive oil is transported into the cell by some surface active substance or by some property of the cell wall. The production of surface active agents by micro-organisms has been shown for some bacteria e.g., for *Pseudomonas fluorescens* (ZOBELL, 1943) and *Vibrio desulfuricans* (LA RIVIÈRE, 1955). RAHN and LEET (1949) suggest, that in case of the inducing of an enzyme by an insoluble substrate, the enzyme may be formed in the cell by a "distance action" of the substrate. However, they did not furnish proof and in the case of lipase it seems to us very unlikely that this actually happens.

Our observations corroborate those of KINGMA BOLTJES (1955), GOODMAN (1950), CUTCHINS and co-workers (1952) and SIERRA (1956). With regard to the *Bacillus* species it has been noted that some strains which did not show any lipase activity, acquired this property if they were incubated during six weeks with Tweens and fats (KINGMA BOLTJES, 1955). The lipase production by *Aspergillus flavus*, *Aspergillus terreus*, and a *Penicillium* species is stimulated when these organisms are grown in the presence of corn oil (GOODMAN, 1950). The lipase production of *Pseudomonas fluorescens* depends upon preferential utilization of certain fatty substrates (CUTCHINS and co-workers, 1952). In recent experiments we found that the lipase of *Pseudomonas aeruginosa* was only produced in those synthetic media which contain certain inducers (SIERRA, 1956).

## Summary.

A simple method to detect lipolytic activity of micro-organisms is described. The formation of lipase is demonstrated by adding water-soluble Tweens to a nutrient medium. Around the colonies with lipolytic activity there appears a well visible halo which is due to crystals of the calcium salt of the fatty acid liberated by lipolysis. The three chief advantages of the use of Tweens are that:

1. there is an optimum contact between the growing cells and the fatty substrate, which is necessary in those cases in which the formation of lipase is adaptive.
2. they can be used in studies on the specificity of the lipase.
3. the results are directly visible.

By examining with this method several strains of bacteria, and comparing it with two other methods, it appeared that in some cases agreement was lacking. Some differences between the three methods are discussed. It is shown that the lipase formation of various strains is adaptive.

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(National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa).

# THREE NEW SPOROGENOUS YEASTS FROM SOIL

by

**J. P. VAN DER WALT**

(Received November 19, 1956).

Three strains of ascosporogenous yeasts have been isolated from South African soil. When examined by the standard procedures of Lodder and Kreger-van Rij (1952) they proved to be typical representatives of the genera *Saccharomyces* (Meyen) Reess, *Pichia* Hansen and *Hansenula* P. et H. Sydow, but they could not be identified with any hitherto described species of these genera. They are accordingly regarded as new species for which the names *Saccharomyces tellustris*, *Pichia terricola* and *Hansenula beijerinckii* are proposed.

## *Saccharomyces tellustris* nov. spec.

### Description.

Growth in malt extract: After 3 days at 25°C., the cells are round to short-oval, (4.0 – 8.0) × (4.3 – 9.5)$\mu$, single or in pairs. After 1 month a sediment is formed.

Growth on malt agar: After 3 days at 25°C., the cells are short-oval to oval, (3.5 – 8.1) × (3.8 – 8.8) $\mu$, single or in pairs. After 1 month at 17°C., the streak culture is yellowish-brown, shiny, with a slight structure near the margin.

Slide cultures: No pseudomycelium, a few elongated cells may be formed.

Sporulation[1]): The spores are large, round with small endogenous oil droplets. Usually 1 per ascus, seldom 2.

---

[1]) Spores were observed on Gorodkowa agar.

| | | | | |
|---|---|---|---|---|
| Fermentation: | glucose | + | maltose | — |
| | galactose | — | lactose | — |
| | saccharose | — | | |

| | | | | |
|---|---|---|---|---|
| Sugar assimilation: | glucose | + | maltose | — |
| | galactose | — | lactose | — |
| | saccharose | — | | |

Assimilation of potassium nitrate: Absent.
Ethanol as sole source of carbon: Growth.
Splitting of arbutin: Weakly positive.

## Discussion.

This strain is brought to the genus *Saccharomyces* since it ferments glucose rapidly, forms no pellicle and does not assimilate nitrate.

Lodder and Kreger-van Rij (1952) recognise four species with round to oval spores which, like the strain from soil, ferment and assimilate only glucose. These are *Sacch. mellis*, *Sacch. bisporus*, *Sacch. baillii* and *Sacch. acidifaciens*. Lodder and Kreger-van Rij (1952) separate these on the basis of cell size in malt extract into the small-celled group *Sacch. mellis* – *Sacch. bisporus* with cell measurements of approximately (2.5 – 5.0) × (3.5 –7) $\mu$ and the remaining two species in a large celled group with cell measurements of approximately (3 – 7) × (5.5 – 15) $\mu$. Since in malt extract the mature cells of the strain from soil measure (4 – 8) × (4.3 – 9.5) $\mu$, it can only be compared to either *Sacch. baillii* or *Sacch. acidifaciens*. It is nevertheless impossible to identify it with either of these species for the following reasons. The strain forms no pseudomycelium whereas both *Sacch. baillii* and *Sacch. acidifaciens* do, although not in a very characteristic form. The growth of *Sacch. baillii* on malt agar is characterised by the occurrence of cells in chains and the curled appearance of the streak culture. In contrast, the cells of the strain from soil never occur in chain-like units; the streak culture is smooth. No better agreement is found when this strain is compared to *Sacch. acidifaciens*. The cells of *Sacch. acidifaciens* in malt extract are characteristically oval to long oval while those of our strain are always round to short oval. Furthermore, *Sacch. acidifaciens* forms 1 – 4 spores per ascus while the strain from soil forms consistently one and exceptionally two spores per ascus.

The above described strain is accordingly regarded as representative of a new species for which the name *Saccharomyces tellustris* is proposed.

## *Pichia terricola* nov. spec.

### DESCRIPTION.

Growth in malt extract: After 3 days cells are short oval to cylindrical (3.8 – 8.8) × (5 – 17) μ, single, in pairs or in short chains. A thin creeping pellicle is formed. After 1 month a sediment and pellicle are present.

Growth on malt agar: After 3 days cells are short oval to cylindrical (3.1 – 8.0) × (5 – 15) μ, single, in pairs or in short chains. Asci are present as well. After 1 month at 17°C. the streak culture is brownish with white sectors, somewhat shiny flat with little wart-like structures. The margin is slightly lobed.

Slide cultures: Pseudomycelium in tree-like formation.

Sporulation[1]: Asci are formed without immediately preceding conjugation. The spores are spherical, smooth with an endogenous oil drop; 1 – 4 spores per ascus.

| | | | | |
|---|---|---|---|---|
| Fermentation: | glucose | + | maltose | — |
| | galactose | — | lactose | — |
| | saccharose | — | | |
| Sugar assimilation: | glucose | + | maltose | — |
| | galactose | — | lactose | — |
| | saccharose | — | | |

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: Growth; a thin pellicle is formed.

Splitting of arbutin: Absent.

### DISCUSSION.

As the organism forms an early creeping pellicle, abundant pseudomycelium and does not assimilate nitrate, it is brought to the genus *Pichia*. The only valid species in the genus hitherto described which ferments only glucose vigorously and assimilates only this sugar, is *Pichia fermentans*. The strain under discussion, however, clearly distinguishes itself from this species. Firstly, its cells are considerably larger than those of *Pichia fermentans* and, secondly, there is a marked difference in their macromorphological characteristics. LODDER and KREGER-VAN RIJ (1952) describe the streak culture on malt agar of *Pichia fermentans* as "yellowish-white, dull, slightly raised or flat, either finely punctate or wrinkled". The streak culture of the strain from soil is somewhat glossy and marked by the occurrence of typical white and brownish sectors. Closer inspection showed that these sectors were due to mutation of the parent strain's sporulating ability, as BEIJERINCK (1897) had found for *Schizosaccharomyces octosporus*. However, where BEIJERINCK observed the brown sectors of *Schiz. octosporus* to be devoid of asci, the reverse was observed for our strain. In this case brown sectors were found actively sporulating while white sectors always contained only very few asci in comparison.

---

[1] Spores were observed on malt agar.

On the ground of these differences the strain from soil is considered as representative of a hitherto unrecognised species for which the name *Pichia terricola* is proposed.

## *Hansenula beijerinckii* nov. spec.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., cells are round to oval, (2.5 – 6.2) × (2.5 – 7.5) $\mu$, single or in pairs. A thin sediment and thick wrinkled pellicle are formed. After 1 month at 17°C. a sediment and pellicle are present.

Growth on malt agar: After 3 days at 25°C., cells are short oval to long oval (1.8 – 5.6) × (2.1 – 7.9) $\mu$, single or in pairs. After 1 month the streak culture is greyish-white, verrucose, somewhat shiny. The margin is slightly crenulated.

Slide cultures: No pseudomycelium is formed.

Sporulation[1]): An isogamous conjugation may precede sporulation. Spores are saturn-shaped with an endogenous oil drop; 1 – 4 spores per ascus.

| | | | | |
|---|---|---|---|---|
| Fermentation: | glucose | + | maltose | — |
| | galactose | — | lactose | — |
| | saccharose | + | raffinose | $^1/_3$ |
| Sugar assimilation: | glucose | + | maltose | + |
| | galactose | + weak or — | lactose | — |
| | saccharose | + | | |

---

[1]) Spores were observed on malt agar.

Assimilation of potassium nitrate: Positive.

Ethanol as sole source of carbon: Growth, a pellicle is formed.

Splitting of arbutin: Positive.

Ester formation: Esters are produced

## DISCUSSION.

The classification of this strain in the genus *Hansenula* is obvious since it possesses the classical biochemical and morphological characteristics of this genus, *viz.*, a positive nitrate assimilation, the formation of esters, the splitting of arbutin, the production of a dull creeping pellicle and saturn-shaped ascospores. This very characteristic ascospore shape places the strain in what WICKERHAM (1951) considers the most recently evolved branch of the genus, consisting, up to the present, of only the species *Hans. californica*, *Hans. suaveolens*, *Hans. mrakii* and *Hans. saturnus*. As the strain has been found to be predominantly diploid, independent of an extraneous vitamin source, to have a marked fermentative ability, to form a well developed creeping pellicle and to have distinctly visible equatorial plates on the ascospores, it may be considered as a rather recently evolved member of this phylogenic branch. When, however, its properties are compared with any of the hitherto described *Hansenula* species, it is found that no species has yet been recorded which form saturn-shaped ascospores, ferments saccharose and raffinose and also assimilates galactose and maltose.On these grounds the strain must be considered as a representative of a hitherto unknown species. For this new species the name *Hansenula beijerinckii* is proposed in honour of M. W. BEIJERINCK (1892) who discovered the most distinctive property of this genus, namely, the assimilation of nitrate.

### *Saccharomyces tellustris* nov. spec.

In musto maltato cellulae ovoideae aut subovoideae (4.0 – 8.0) × (4.3 – 9.5) $\mu$, singulae aut binae. Sedimentum formatur.

In agaro maltato cellulae subovoideae aut ovoideae (3.5 – 8.1) × (3.8 – 8.8) $\mu$, singulae aut binae. Cultura (post unum mensem 17°C.) flavifusca, nitida, in parte extrema parum crispulata.

Pseudomycelium nullum.

Ascosporae rotundae, parvos globulos olei continent; una perraro duae in asco.

Fermentatio glucosi solum. In medio minerali solum cum glucoso crescitur. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico crescitur. Arbutinum finditur (paulum).

Isolata ex terra.

### *Pichia terricola* nov. spec.

In musto maltato cellulae subovoideae aut cylindricae (3.8 – 8.8) × (5 – 17) $\mu$, singulae, binae aut catenatae. Post dies 3 pellicula, non nitida, tenuis, sursum repens, formatur. Sedimentum.

In agaro maltato cellulae ovoideae aut cylindricae (3.1 – 8.0) × (5 – 15) $\mu$, singulae, binae aut catenatae. Asci. Cultura (post unum mensem 17°C.) parum nitens cum albis et fuscis sectoribus, plana cum verrucis parvis. Margine paulum lobiformo.

Pseudomycelium abundat. Cellulae pseudomycelii et blastosporae longovoideae cylindricaque in catenatis ramosis.

Ascosporae rotundae, plerumque globulos olei continent, 1–4 in asco.

Fermentatio solum glucosi. In medio minerali solum cum glucoso crescitur. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico crescitur, pellicula formatur. Arbutinum non finditur.

Isolata ex terra.

### *Hansenula beijerinckii* nov. spec.

In musto maltato cellulae rotundae aut ovoideae (2.5 – 6.2) × (2.5 – 7.5) $\mu$, singulae aut binae. Post dies 3 pellicula, non nitida, crispulata, sursum repens, formatur. Sedimentum.

In agaro maltato cellulae ovoideae aut longovoideae (1.8 – 5.6) × (2.1 – 7.9) $\mu$, singulae aut binae. Cultura (post unum mensem 17°C.) griseola alba, acclivis, parum nitens, cum verrucis parvis, margine ad morum crenellato.

Pseudomycelium nullum.

Copulatio cellularum aequarum conformationi ascorum interdum praecedit. Ascosporae saturniformae, plerumque parvos globulos olei continent; 1 – 4 in asco.

Fermentatio glucosi, sacchari, raffinosi pro tertia parte. Crescitur in medio minerali cum glucoso, cum galactoso (paulum), cum saccharo, cum maltoso. Nitras kalicus assimilatur. In medio minerali cum

alcohole aethylico crescitur, pellicula formatur. Arbutinum finditur. Esterae formantur.

Isolata ex terra.

## Acknowledgement.

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# THE ORGANIZATION OF THE NUCLEUS IN *BACILLUS MEGATHERIUM*

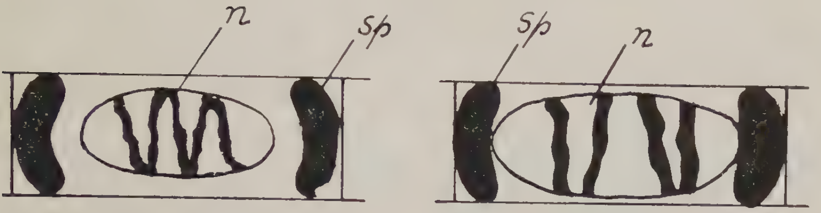
by

**AKIRA YUASA**

(Received August 10, 1956).

Air-dried smears of *Bacillus megatherium* were stained with one per cent crystal violet (either directly or after pre-treatment for twenty minutes with ten per cent perchloric acid). Threads of granules of chromatin were observed to occupy a vacuole of variable size (figure 1) delimited by the densely stained cytoplasm. It is the purpose of the present communication to identify this structure as the nuclear vacuole and to suggest that each vegetative cell of *Bacillus megatherium* contains a single nucleus. This view, which was originally suggested by FITZ-JAMES (1954) and ROBINOW (1953), is supported (a) by the appearance of the nuclear vacuole, (b) by the fact that at the termination of mitosis two masses of chromatin appear in the center of the dividing cell (YUASA, 1956a, YUASA, 1956b) (figure 2), and (c) by the fact that (personal communication) Dr S. SPIEGELMAN has been able to isolate a single large nucleus from each cell of *Bacillus megatherium*. This concept differs from that of DELAMATER (1953), DELAMATER and MUDD (1951) and HUNTER-SZYBALSKA, SZYBALSKI and DELAMATER (1956), who hold that the cell of *Bacillus megatherium* is multinucleate. DELAMATER has inferred that each actively growing vegetative cell contains from 2 to 4 nuclei each with two or three chromosomes. The data presented herewith are interpreted as follows: Before mitosis each nuclear vacuole contains two chromosomes which divide to produce four just prior to cell division.

In addition to the chromosomes, another basophilic structure is present in the cell. HUNTER and DELAMATER (1952, 1955) and DELAMATER and HUNTER (1952) have distinguished the central



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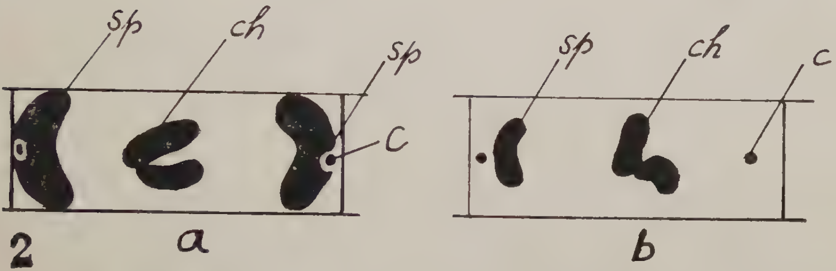

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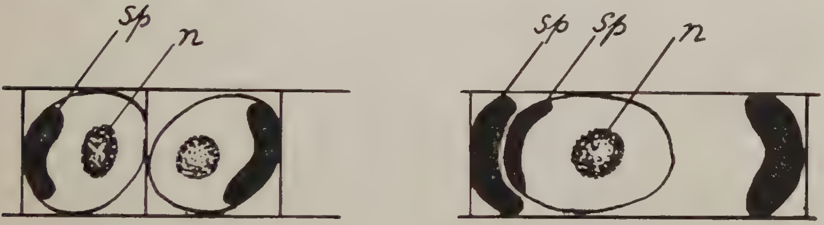

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Fig. 1. Resting cell. *n*, nucleus; *sp*, spindle.

Fig. 2. *a*. Schematic representation of a cell in mitosis. *b*. after treatment with 2 N HCl for 24 hours; stained with gentian violet. *c*, centriole; *ch*, chromosome; *sp*, spindle.

Fig. 3. Sporulation. *n*, nucleus; *sp*, spindle.

chromatinic body (the chromosomes) from the peripheral chromatinic body in the spore of *Bacillus megatherium*. KEIGLER and SMITH (1954) found both a central and a peripheral chromatinic body in spores of *Bacillus cereus* grown on a dilute casein hydrolyzate medium. The peripheral chromatinic body is identified as the spindle, and it is inferred that the vegetative cell of *Bacillus megatherium* contains a spindle at each pole. The spindle resists solution after exposure to 10 per cent perchloric acid, 5 per cent KOH, or 1 N HCl for 20 minutes. Longer treatment (at room temperature) with 5 per cent KOH, 2 N HCl or 1 N HCl (60 C.), destroys the spindle and reveals the centriole concealed in the spindle. Treatment with

2 N HCl for 30 minutes (at room temperature) also destroys the spindle revealing the centriole. DeLamater (1951;1952) and Hunter-Szybalski, Szybalski and DeLamater (1956) observed the centriole after treatment with 1 N HCl (60 C.) (which destroys the spindle). When spores are formed (figure 3) the cell is either (a) divided into smaller cells, each of which is transformed into a spore (containing a nucleus and a spindle) or (b) a single large spore is formed between two spindles in the undivided vegetative cell, leaving one spindle inside the spore and the other in the epiplasm.

The writer wishes to express his sincere thanks to Professor Dr C. C. Lindegren for making available the facilities which made it possible to carry on this investigation.

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# A SIMPLE KEY FOR THE BIOCHEMICAL DETERMINATION OF *ENTEROBACTERIACEAE*

by

**J. G. HEYL**

(Received November 12, 1956).

The serological identification of the pathogenic enteric bacteria favoured by a greater stability and specificity of the diagnostic characteristics is perhaps more useful than any other method. In many cases, however, as for instance in the occurrence of common antigens in different groups or types of *Enterobacteriaceae*, confirmation of the serological typing by investigation of the biochemical properties may be necessary.

The variability of some of these properties found in strains belonging to one group or the occurrence of different biochemical types within one serological type requires in general the investigation of a great number of biochemical and cultural characteristics to obtain sufficient data for the identification of the isolated cultures.

In the present state of perfection in typing *Enterobacteriaceae* there exists, without loss of security, a tendency to decrease the number of necessary biochemical tests and in many cases these attempts have been more or less successful.

Buttiaux *et al.* (1956) have worked out a scheme for identification of the non-lactose fermenting *Enterobacteriaceae* based upon 8 cultural and biochemical properties.

Kauffmann and Petersen (1956) proposed the utilisation of organic acids as a criterion in differentiating *Enterobacteriaceae*.

However, many of the methods described often have the drawback of being too complicated in the composition or in the preparation of the media required for general use and their application is restricted to laboratories, specialised in the research of this group of pathogenic bacteria.

On the following pages a scheme is presented for the determination of groups of *Enterobacteriaceae* based, it is true, upon many but otherwise fairly simple biochemical and cultural properties.

The proposal of KAUFFMANN, EDWARDS and EWING (1956) to avoid the Linnaean ,,species'' concept in the classification of the *Enterobacteriaceae* and to subdivide these bacteria for practical purposes into biochemical groups and subgroups is followed in the discussion of the groups of micro-organisms concerned.

In the same communication of these authors the different behaviour of groups and types in biochemical tests are designated as follows:

+ = positive
(+) = delayed positive
× = late and irregularly positive or negative
— = negative
d = different biochemical types within a single sero-type.

It may be mentioned here that, in using the following tables for identification, the key given above will not be repeated.

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## MEDIA AND TECHNIQUES.

The following media and techniques are recommended for carrying out the biochemical tests.

### 1. Fermentation of carbohydrates.

| | | |
|---|---|---|
| Bacto peptone | 10 g | |
| Sodium chloride | 5 g | |
| Distilled water | 1000 ml | pH adjusted at 7.6. |

After dissolving the ingredients 10 ml of the Andrade-indicator is added. Sterilize for 15 minutes at 115° C. After sterilization 1% of the carbohydrate to be tested is added.

Andrade-indicator:

| | |
|---|---|
| Distilled water | 100 ml |
| N NaOH | 16 ml |
| Acid fuchsin | 0.5 g |

The tests have to be read daily for 10–12 days.

### 2. Indole-base.

| | | |
|---|---|---|
| Bacto-tryptone | 10 g | |
| Sodium chloride | 5 g | |
| Distilled water | 1000 ml | pH 6.8 — 7.2 |

Sterilize for 15 minutes at 120° C.

After incubation for 24 — 48 hours at 37° C. the test is carried out by adding about 0.2 — 0.3 ml of the reagent of Ehrlich-Böhm. The reagent is prepared by dissolving 5 g para-dimethyl-amido benzaldehyd in 75 ml N amyl alcohol and adding to this solution 25 ml concentrated (38%) HCl.

### 3. Methyl-red and Voges-Proskauer test.

| | |
|---|---|
| Bacto peptone | 5 g |
| Potassium phosphate | 5 g |
| Distilled water | 975 ml |

Boil to dissolve the ingredients, filter through paper and sterilize the filtrate for 20 minutes at 115° C. After sterilization 25 ml of a sterilized 25% solution of dextrose are added.

Tubes filled with about 10 ml of this substrate are inoculated and incubated for 3 days at 37° C. For the Voges-Proskauer test (Barritt's modification) 2 ml of the culture are placed into a second tube. To these 2 ml culture 1.2 ml of a 5% alpha-naphthol solution in ethanol 96% and 0.4 ml of a 40% KOH solution are added. To the rest of the culture 10 drops of a 0.25% alcoholic methyl-red solution are given.

**4. Citrate medium according to Koser.**

| | | |
|---|---|---|
| Sodium chloride | 5.0 g | |
| Sodium ammonium phosphate | 1.0 g | |
| Monopotassium phosphate | 1.0 g | |
| Magnesium sulfate | 0.2 g | |
| Sodium citrate | 3.0 g | |
| Distilled water | 1000 ml | pH at 6.8 |

**5. Medium for $H_2S$ reduction.**

| | | |
|---|---|---|
| Beef broth | 1000 ml | |
| Agar | 5.0 g | |
| Sodium sulphite | 40.0 g | |
| Dextrose | 10.0 g | |
| Ferro-sulphate | 0.8 g | pH at 7.6 |

**6. Urea agar base according to Christensen.**

| | |
|---|---|
| Bacto peptone | 1.0 g |
| Sodium chloride | 5.0 g |
| Monopotassium phosphate | 2.0 g |
| Phenol-red | 0.012 g |
| Distilled water | 1000 ml |

Dissolve the ingredients by boiling; adjust pH at 6.9 and autoclave 15 minutes at 120° C. After sterilization add 1 g dextrose and 20 g urea in Seitz-filtered solutions.

**7. Medium for the KCN test according to Møller.**

| | |
|---|---|
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Monopotassium phosphate | 0.225 g |
| Sodium phosphate | 5.64 g |
| Distilled water | 1000 ml |

This medium is autoclaved for 20 minutes at 115° C.; add to the cold medium 15 ml of a 0.5% solution ot KCN in distilled water. Divide the medium in quantities of 1 ml in small tubes, which are quickly stoppered with sterilized paraffinated corks or with rubber stoppers after adding a thin layer of sterilized paraffin to the medium. The inoculated tubes must be read daily for 4 days, incubated at 37° C.

**8. Leifson's malonate medium according to Shaw (combined with Henriksen's phenylalanin test).**

| | |
|---|---|
| Ammonium sulfate | 2.0 g |
| Potassium phosphate | 0.6 g |
| Monopotassium phosphate | 0.4 g |
| Sodium chloride | 2.0 g |
| Sodium malonate | 3.0 g |

| | |
|---|---|
| DL-phenylalanin | 2.0 g |
| Yeast extract | 1.0 g |
| Bromo-thymol blue | 0.025 g |

Cultures are incubated overnight at 37° C.; an alkaline reaction is produced by bacteria using malonate as a source of carbon.

## SURVEY OF THE GROUPS AND TYPES OF *Enterobacteriaceae*.

The nomenclature of groups and types given in Table 1 is as far as possible adjusted at the practice required in clinical laboratory diagnosis.

TABLE 1.

Provisional identification of groups of *Enterobacteriaceae*.

| Groups of *Enterobacteriaceae* | Glucose | Gas | Lactose | Mannitol | Indole | Methyl-red | Voges-Proskauer | Sodium citrate | Urea | $H_2S$ | KCN-test |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Escherichia | + | + | + | + | +/— | +/— | — | +/— | — | — | — |
| Paracolobactrum | + | + | (+) | + | +/— | +/— | +/— | +/— | +/— | — | +/— |
| Klebsiella/Cloaca | + | + | + | + | — | +/— | +/— | +/— | +/— | — | + |
| Salmonella/Arizona | + | + | (+)/— | + | — | + | — | + | — | +/— | — |
| Ballerup-Bethesda | + | + | (+)/— | + | — | + | — | + | — | + | + |
| Proteus | + | +/— | — | +/— | +/— | +/— | — | +/— | + | +/— | + |
| Providencia | + | +/— | — | +/— | + | + | — | +/— | — | — | + |
| Hafnia | + | +/— | — | + | — | + | +/— | +/— | — | (+) | + |
| Erwinia | + | + | + | + | — | + | — | + | — | — | + |
| Serratia | + | — | (+)/— | + | — | +/— | +/— | +/— | — | — | + |
| Shigella/Alcalescens | + | — | (+)/— | +/— | +/— | +/— | — | — | — | — | — |
| B 5 w (*B. anitratum*) | + | — | (+)/— | — | — | — | — | + | — | — | — |

*Salmonella typhi*: no gas
*Shigella flexneri* type 6: with gas

| | |
|---|---|
| Dextrose: +, gas: + | 2 |
| Dextrose: +, gas: — | 3 |
| 2. mannitol: +, indole: + | |
| lactose: +, KCN: — | Escherichia group |
| lactose: (+), KCN: — | Paracolobactrum group |
| lactose: —, KCN: + | Providencia group |
| mannitol: +, indole: — | |
| lactose: +, KCN: + | |
| $H_2S$: + | Ballerup-Bethesda group |
| $H_2S$: — | |
| inositol: + | Klebsiella group |

| | |
|---|---|
| inositol: — | Cloaca group |
| lactose: +, KCN: — | |
| $H_2S$: + | Arizona group |
| $H_2S$: — | |
| maltose: +, sorbitol: + | Escherichia group |
| maltose: —, sorbitol: — | Erwinia group |
| lactose: (+), KCN: + | |
| $H_2S$: + | Ballerup-Bethesda group |
| $H_2S$: — | Paracolobactrum- and Cloaca group |
| lactose: (+), KCN: — | Arizona group |
| $H_2S$: + | |
| lactose: —, KCN: + | Ballerup-Bethesda group |
| $H_2S$: +, sorbitol: + | Hafnia group |
| $H_2S$: (+), sorbitol: — | Klebsiella-and Cloaca group |
| $H_2S$: —, sorbitol: + | |
| lactose: —, KCN: — | |
| $H_2S$: + | Salmonella group |
| dulcitol: +, gelatin: — | Arizona group |
| dulcitol: —, gelatin: + | Shigella group |
| $H_2S$: — | |
| mannitol: —, indole: + | |
| lactose: —, KCN: + | Proteus group |
| urea: + | Providencia group |
| urea: — | |
| mannitol: —, indole: — | |
| lactose: —, KCN: + | Proteus group |
| urea: + | Shigella group |
| lactose: —, KCN: — | |
| | |
| 3. mannitol: +, indole: + | Alcalescens-dispar group |
| lactose: +/(+), KCN: — | |
| lactose: —, KCN: + | Proteus group |
| urea: + | Providencia group |
| urea: — | |
| lactose: —, KCN: — | Alcalescens-dispar group |
| Christensen citrate alcalinized | Shigella group |
| not alcalinized | |
| mannitol: +, indole: — | Serratia group |
| lactose: (+), KCN: + | |

TABLE 2.

Biochemical reactions of *E. coli* and *E. intermedium*.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | + | + | + | + | +/— | + | + | + | + | +/— | + | — | — | +/— | + | + | — | — | — | — | — | — | +/— |
| *E. intermedium* | + | + | + | + | +/— | +/— | + | + | + | +/— | + | — | — | — | +/— | + | — | + | — | — | — | — | +/— |

| | |
|---|---|
| lactose: (+), KCN: — | Shigella group |
| lactose: —, KCN: + | Hafnia group |
| lactose: —, KCN: — | Shigella group |
| mannitol: —, indole: + | |
| lactose: —, KCN: + | Providencia group |
| lactose: —, KCN: — | Shigella group |
| mannitol: —, indole: — | |
| lactose: —, KCN: — | |
| Na-citrate: + | B.5.w. group |
| Na-citrate: — | Shigella group |

**Escherichia group.**

As shown in table 2 the Escherichia group is biochemically represented by two subgroups: *E. coli* and *E. intermedium.* KAUFFMANN (1954) serologically divides the Escherichia group into the subgroups *E. coli* and *E. freundii,* the latter including the Ballerup-Bethesda group. In one of his latest communications KAUFFMANN (1956) proposed the name *Citrobacter* comprehending *E. freundii* and Ballerup-Bethesda. In the scheme presented here the strains fermenting promptly lactose, forming $H_2S$ and with positive KCN-test, known as *E. freundii,* are, for practical purposes, set apart from the Escherichia group and placed into the Bethesda group.

*Escherichia coli.*

*E. coli* strains promptly attack nearly all fermentable substances usually added to the medium, mostly with the formation of gas. Anaerogenic strains, however, may occur. Typical biochemical variants in the coli-group can be recognized by their ability to split sucrose and salicin (table 3).

TABLE 3.

Biochemical variants of *Escherichia coli.*

| | sucrose | salicin |
|---|---|---|
| *E. coli* var. *neapolitana* | + | + |
| *E. coli* var. *communior* | + | — |
| *E. coli* var. *commune* | — | + |
| *E. coli* var. *acidi-lactici* | — | — |

Most, but not all strains connected with epidemic infantile gastro-enteritis (serological types O 26, O 55, O 86, O 111 a.o.) belong to *E. coli* var. *neapolitana.*

The biochemical differentiation of the most important serotypes are summarized in table 4.

TABLE 4.

Biochemical behaviour of *E. coli* strains, connected with epidemic infantile gastro-enteritis.

| Group of *E. coli* | sorbose | sucrose | maltose | dulcitol | sorbitol |
|---|---|---|---|---|---|
| O group 26 B 6 | + | (+) | + | (+) | + |
| O group 26 B 6.11 1) | + | (+) | + | — | + |
| O group 55 B 5.6 | + | + | (+) | + | × |
| O group 86 B 7 | + | + | + | (+) | — |
| O group 111 B4.—4.2 | — | + | + | (+) | + |
| O group 111 B4.12 | — | — | + | (+) | + |

1) aerogenic or anaerogenic.

For further details see:

LE MINOR, S. et L., NICOLLE, P. et BUTTIAUX, R. 1954. Etudes sur les *Escherichia coli* isolés au cours des gastro-entéritis infantiles. Ann. Inst. Pasteur **86**, 204.

*Escherichia intermedium.*

BERGEY describes the imvic-formula of *E. intermedium* as +/—, +, —, +, being intermediate between Escherichia (+ + — —) and Klebsiella (— — + +).

This suggests to consider *E. intermedium* as a single species with stabile properties instead of a group of strongly interrelated bacteria with different biochemical behaviour mainly in the composition of the imvic-reaction. In reality 14 of the 16 combinations of the imvic formula are possible within the intermedium-group. In the course of an investigation of 500 cultures of coliform bacteria isolated from normal human feces we encountered 12 of these 14 combinations, divided as indicated in table 5.

TABLE 5.

| Imvic | % | Imvic | % | Imvic | % | Imvic | % |
|---|---|---|---|---|---|---|---|
| + + + + | 2.60 | + — — + | — | — + — + | 7.00 | — + + + | 1.40 |
| + + + — | 1.40 | + — + + | 0.95 | — — + — | 0.50 | — — + + | 2.60 |
| + + — — | 64.40 | + + — + | 5.80 | — + — — | 8.00 | — — — + | 0.95 |
| + — — — | 0.70 | + — + — | — | — + + — | 2.80 | — — — — | 0.90 |

### Erwinia group and Serratia group.

With regard to their biochemical behaviour *Erwinia* and *Serratia* strains are considered as belonging to the *Enterobacteriaceae.* KAUFFMANN, who examined serologically a number of strains of these groups found an antigenic relationship with the Escherichia group. Further biochemical and serological investigations will be necessary to obtain evidence for the real taxonomic position of these more or less heterogeneous groups of bacteria. The biochemical reactions of Erwinia and Serratia are summarized in table 6.

### Paracolobactrum group.

The biochemical behaviour of strains of *Paracolobactrum* (named by BORMAN) is identical to that of types of the Escherichia and Klebsiella group with the exception of a characteristically delayed fermentation of lactose. Some authors do not accept a demarcation, only on the base of a delayed splitting of lactose, between this group and the Escherichia and Klebsiella group, as those cultures otherwise behave like typical strains of the two groups mentioned above, and include *Paracolobactrum* into these groups. Mainly on differences in the imvic-reaction the Paracolobactrum group is divided into 3 subgroups, namely: *P. coliforme, P. intermedium, P. aerogenoides.*

What is stated above as to *Escherichia intermedium* being a group of interrelated bacteria between *Escherichia* and *Klebsiella* may be proposed in the same way as to *Paracolobactrum intermedium* being an intermediate group of bacteria between *P. coliforme* and *P. aerogenoides.*

### References.

BORMAN, E. K., STUART, C. A. and WHEELER, K. N. 1944. Taxonomy of the family *Enterobacteriaceae.* J. of Bact. **48**, 351.

SCHAUB, I. G. 1948. The cultural differentiation of paracolon bacteria. Bull. John Hopkins Hosp. **83**, 367.

### Klebsiella group and Cloaca group.

On account of the corresponding biochemical and serological properties no difference is made nowadays between *Klebsiella* and *Aerobacter*, both being placed into one group: the Klebsiella group. The placing of *Aerobacter* (type culture *Aerobacter aerogenes*) into the Klebsiella group resulted in a difficult taxonomic position of

## TABLE 6.

Biochemical reactions of *Erwinia* and *Serratia* strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Erwinia* | + | + | + | — | + | (+) | (+) | (+) | + | — | — | × | — | + | — | + | — | + | — | — | + | — | +/— |
| *Serratia* | + | — | (+)— | + | + | — | +/— | +/— | + | — | +/— | (+) | (+) | + | — | +/— | +/— | +/— | — | — | + | — | + |

## TABLE 7.

Biochemical reactions of *Paracolobactrum* strains.

| Subgroups of Paracolobactrum | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. coliforme* | + | + | (+) | + | +/— | + | + | + | + | — | + | — | — | + | + | + | — | — | — | — | — | — | +/— |
| *P. intermedium* | + | + | (+) | + | +/— | + | + | + | + | — | + | — | — | — | +/— | + | — | +/— | +/— | — | — | + | +/— |
| *P. aerogenoides* | + | + | (+) | + | + | + | + | + | + | — | + | +/— | +/— | + | — | — | + | + | + | — | — | + | +/— |

*Aerobacter cloacae* because of distinct cultural and biochemical differences of strains belonging to this type and *Klebsiella* types. The problem was solved by framing a separate group: the Cloaca-group, enclosing all types formerly known as *Aerobacter cloacae*. The biochemical reactions of typical *Klebsiella* and *Cloaca* strains are given in table 8.

*Klebsiella* strains attack nearly all fermentable carbohydrates. Motility and liquefaction of gelatin distinguish *Cloaca* strains from *Klebsiella* strains. As in all groups of *Enterobacteriaceae* intermediate types and deviations of the normal biochemical characteristics will be encountered. Anaerogenic strains, which are methyl-red positive, Voges-Proskauer negative and citrate and urea negative belong to the "rhinoscleromatis type".

The biochemical reactions of the so-called „ozaena-types" are generally intermediate between those of the "pneumoniae-types" and the "rhinoscleromatis types".

Aberrant biochemical behaviour of *Klebsiella* strains may sometimes interfere with the differentiation of *Klebsiella* and *Escherichia* types. In that case the fermenting of inositol and the positive results of the KCN-test by *Klebsiella* are typical differences.

### Cloaca-group.

As mentioned above the name is derived from *Aerobacter cloacae*. The biochemical activity of *Cloaca* strains is comparable with those of the Klebsiella and Escherichia group. Contrary to *E. coli* types *Cloaca* cultures slowly liquefy gelatin and are positive in the KCN-test.

### References.

Edwards, P. R. and Fife, E. M. 1955. Studies on the Klebsiella-Aerobacter group of bacteria. J. Bact. **70**, 382.

Brooke, M. S., 1951. Biochemical investigation on certain urinary strains of *Enterobacteriaceae*. Acta path. microb. Scand. **29**, 1.

Brooke, M. S. 1954. The differentiation of *Aerobacter aerogenes* and *Aerobacter cloacae*. J. Bact. **66**, 721.

Ørskov, I. 1955. The biochemical properties of *Klebsiella* strains. Acta path. microb. Scand. **37**, 353.

### Salmonella group and Arizona group.

Since a biochemical differentiation of the known *Salmonella* types is hardly possible and as the serological typing has been rendered

## TABLE 8.

Biochemical reactions of typical *Klebsiella* and *Cloaca* strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Klebsiella* | + | + | + | + | + | + | + | + | + | +/— | + | + | + | + | — | +/— | +/— | +/— | + | — | — | + | — |
| *Cloaca* | + | + | + | + | + | +/— | + | +/— | + | — | + | +/— | +/— | +/— | — | +/— | + | + | +/— | — | + | + | + |

## TABLE 9.

Biochemical properties of *Salmonella* and *Arizona* strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Salmonella* | + | + | — | + | — | +/— | + | + | + | + | + | +/— | — | — | — | + | — | + | — | +/— | — | — | +/— |
| *Arizona* | + | + | (+)/— | + | — | + | + | + | + | — | + | — | — | — | — | + | — | + | — | +/— | + | — | + |

*S. typhi*: no gas.

very reliable there is no need for the former one and a detailed description of the biochemical properties of these types can be omitted.

Strains of the Arizona group originally named *Salmonella arizona*, are closely related to *Salmonella* types. Comprehensive serological studies, in particular by Edwards and his collaborators, have led to the concept of a separate Arizona group.

The main differences in biochemical behaviour between *Salmonella* and *Arizona* types are a delayed fermentation of lactose by most *Arizona* strains, further non-splitting of dulcitol and liquefaction of gelatin by members of the same group. Culturally the types of the Arizona group can be distinguished from *Salmonella* types by their alkaline reaction in Leifson's malonate medium. The normal biochemical reactions of strains of the two groups are as indicated in table 9.

Kauffmann mentions several deviations of the normal reactions; the most important of these being:

1. slowly lactose-fermenting *Salmonella* variants,
2. non- or delayed maltose splitting strains,
3. anaerogenic strains,
4. indole-positive variants.

## References.

Kauffmann, F. 1954. *Enterobacteriaceae*. 2nd. Ed., Munksgaard, Copenhagen.

Edwards, P. R., West, M. G. and Bruner D. W. 1946. Arizona group of paracolon bacteria. Kentucky Agric. Exp. Stat. Lexington Bull. 499 (See J. Inf. dis. **81**, 19, 1947).

Shaw, C. 1956. Distinction between Salmonella and Arizona by Leifson's sodium malonate medium. Int. Bull. Bact. Nom. Tax. **6**, 1.

### Ballerup-Bethesda group.

The Ballerup group (type culture formerly known as *Salmonella ballerup*) and the Bethesda group are biochemically much alike and serologically closely related.

As many paracolon bacteria strains of the Ballerup-Bethesda group are characterized by a mostly delayed fermentation of lactose. On the basis of common antigens Kauffmann considers *Escherichia freundii* more or less as a type culture for the Bethesda group. A striking difference between the Salmonella-Arizona groups

## TABLE 10.

Biochemical behaviour of Ballerup-Bethesda strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ballerup-Bethesda | + | + | (+)/— | + | (+)/— | + | + | + | + | +/— | + | — | — | (+)/— | — | + | — | + | — | + | — | + | + |

and the Bethesda-Ballerup group is the positive reaction in the KCN-test by the latter. For further details of the biochemical properties the scheme of table 10 may be given.

### References:

EDWARDS, P. R., WEST, M. G. and BRUNER, D. W. 1948. Antigenic study of a group of paracolon bacteria. (Bethesda group). J. Bact. **55, 711.**

GALBRAITH, E. L. and CROSSLEY, V. M. 1953. The Bethesda group of paracolon bacteria. Can. J. Publ. Health **44, 172.**

BRUNER, D. W. and HOPSON, A. S. 1949. Serological classification of the Ballerup group of paracolon bacteria. J. inf. dis. **85, 290.**

BUTTIAUX, R., 1952. Distinction rapide entre Salmonella et bacilles paracoli du groupe Bethesda-Ballerup. Ann. Inst. Pasteur **83, 156.**

## Proteus group.

The Proteus group is subdivided into 4 subgroups, which can be distinguished easily by their different biochemical behaviour (table 11).

TABLE 11.

Biochemical differentiation of Proteus subgroups.

| Subgroups of Proteus | gas | maltose | xylose | mannitol | indole | Na-citrate | $H_2S$ |
|---|---|---|---|---|---|---|---|
| *Proteus vulgaris* | + | + | + | — | + | — | + |
| *Proteus mirabilis* | + | — | + | — | — | — | + |
| *Proteus morganii* | + | — | — | — | + | — | (+) |
| *Proteus rettgeri* | — | — | — | + | + | + | — |

A complete survey of the biochemical reactions of the Proteus subgroups is given in table 12.

According to PERCH, referred by KAUFFMANN in his book "*Enterobacteriaceae*", minor deviations of the normal scheme of reactions may occur. KAUFFMANN (1956) accepted a restriction of the name "Proteus" for the subgroups *P. vulgaris* and *P. mirabilis.*

The subgroups *P. morganii* and *P. rettgeri* are more or less set apart as new genera, respectively named *Morganella* and *Rettgerella.*

## TABLE 12.

Biochemical behaviour of Proteus subgroups.

| Subgroups of Proteus | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. vulgaris* | + | + | — | + | + | — | — | + | — | — | — | — | — | + | + | + | — | — | + | + | + | + | + |
| *P. mirabilis* | + | + | — | — | × | — | — | + | — | — | — | — | — | × | — | + | — | — | + | + | + | + | + |
| *P. morganii* | + | + | — | — | +/— | — | — | — | — | — | — | — | — | — | + | + | — | — | + | (+) | — | + | + |
| *P. rettgeri* | + | — | — | — | +/— | +/— | +/— | — | + | — | — | (+) | (+) | +/— | + | +/— | — | + | + | — | — | + | + |

## References.

BUTTIAUX, R. 1954. Les propriétés biochimiques charactéristiques du genre *Proteus*. Inclusion souhaitable des Providencias dans celui-ci. Ann. Inst. Pasteur **87**, 375.

KAUFFMANN, F. 1956. Zur biochemischen und serologischen Gruppen- und Typen Einteilung der Enterobacteriaceae. Zentralbl. f. Bakt. I, **165**, 344.

### Providencia group.

Biochemically the Providencia group is closely related to the Proteus group and to the Shigella group. It is a heterogeneous group of bacteria with many differences in their biochemical behaviour. About 50% of the strains are anaerogenic. Aerogenic cultures are usually adonitol-positive and inositol-negative; anaerogenic strains adonitol-negative and inositol-positive. According to EWING, aerogenic cultures generally belong to his biochemical group 1 and anaerogenic strains to biochemical group 2.

Summarizing the results of biochemical investigations of this group by EWING, by KAUFFMANN and by BUTTIAUX the scheme of table 13 can be presented.

## References.

BUTTIAUX, H., FRENOY, R. et MORIAMEZ, J. 1955. Les caractères biochimiques du genre *Providencia*. Ann. Inst. Past. de Lille **6**, 62.

RIDGE, L. E. L. and THOMAS, M. E. M. 1955. Infections with the Providence type of paracolon bacillus in a residential nursery. Jrnl. path. bact. **69**, 335.

EWING, W. H., TANNER, K. E. and DENNARD, D. A. 1954. The Providence group; an intermediate group of enteric bacteria. J. inf. Dis. **94**, 134.

### Hafnia group.

Orginally and recently named and described by MØLLER. The type-culture is called: *Hafnia alvei*.

KAUFFMANN draws the attention to the importance of this group because of the fact, that many, "doubtful" cultures of *Enterobacteriaceae* may belong to *Hafnia*, whose biochemical characteristics are given in table 14.

It has to be noted, that cultures tested at low temperature, f.i. at 20° C, are methyl-red negative and Voges-Proskauer and citrate positive.

## TABLE 13.

Biochemical reactions of *Providencia* strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Providencia* | + | +/— | — | — | × | — | — | — | +/— | — | × | +/— | +/— | +/— | + | + | — | +/— | — | — | — | + | + |

## TABLE 14.

Biochemical reactions of *Hafnia* strains.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hafnia alvei* | + | +/— | — | + | +/— | + | +/— | + | + | — | — | — | — | +/— | — | + | +/— | (+)/— | — | (+) | — | + | +/— |

### References.

MøLLER, V. 1954. Distribution of amino acid decarboxylases in *Enterobacteriaceae*. Acta. path. microb. Scand. **35**, **259**.
KAUFFMANN, F. 1954. *Enterobacteriaceae*. 2nd. Ed. Munksgaard, Copenhagen.

**Shigella group and Alcalescens group.**

The combination of these two groups surely does not imply the supposition of a relationship between them. According to KAUFFMANN the Alcalescens-dispar group is strongly related to the Escherichia group and not to the Shigella group. It is the corresponding biochemical behaviour, as shown in table 15, that placed the two groups here together.

Nevertheless *Alcalescens-dispar* types and *Shigella* types can easily be distinguished from each other, namely: All *Alcalescens-dispar* types split maltose, rhamnose, arabinose and xylose; only an occasional *Shigella* type does. All strains belonging to the Alcalescens-dispar group alcalinize Christensen's citrate medium and no Shigella type does.

In the Alcalescens-dispar group and in the Shigella group the occurrence of deviations in biochemical behaviour of strains belonging to one serological type is a real disadvantage for a complete identification of these types. As far as possible a scheme for the differentiation of strains, based upon normal biochemical reactions, can be given as follows:

Alcalescens-dispar group
mannitol: +, indole: +
lactose: +, KCN: —
rhamnose: + , dulcitol: —, sucrose: — O group 6
rhamnose: + , dulcitol: +/—, sucrose: (+) O group 5
rhamnose: + , dulcitol: +/—, sucrose: +/— O group 3
rhamnose: (+), dulcitol: (+), sucrose: + O group 7
lactose: (+), KCN: —
rhamnose: + , dulcitol: —, sucrose: (+) O group 4
rhamnose: + , dulcitol: +, sucrose: — O group 8
lactose: —, KCN: —
rhamnose: + , dulcitol: +, sucrose: — O group 1
rhamnose: + , dulcitol: —, sucrose: — O group 2
rhamnose: + , dulcitol: +/—, sucrose: +/— O group 3

## TABLE 15.

Biochemical behaviour of Alcalescens-dispar and Shigella types.

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A-D group | + | — | (+)/— | + | (+)/— | + | + | + | + | +/— | +/— | — | — | — | + | + | — | — | — | — | — | — | — |
| Shigella | + | — | (+)/— | +/— | (+)/— | +/— | +/— | +/— | +/— | (+)/— | +/— | — | — | — | +/— | +/— | — | — | — | — | — | — | — |

*Shigella flexneri* type 6: with gas.

Shigella group.

Dextrose: +, gas: — 2

Dextrose: +, gas: + 3

2. mannitol: +, indole: +

- xylose: +/(+)
  - arabinose: +/(+)
    - rhamnose: +,
      - maltose: + , sorbitol: + Serotype 792
      - maltose: (+), sorbitol: — *Sh. boydii* type 11
    - rhamnose: —
      - maltose: (+), sorbitol: + *Sh. boydii* type 7
      - maltose: + , sorbitol: + *Sh. boydii* type 5
- xylose: —
  - arabinose: +/(+)
    - rhamnose: +/(+)
      - maltose: +/—, sorbitol: + *Sh. flexneri* type 3
      - maltose: (+), sorbitol: +/— *Sh. flexneri* type 4
    - rhamnose: —
      - maltose: +/—, sorbitol: + *Sh. flexneri* type 3
      - maltose: +/—, sorbitol: — *Sh. flexneri* type 2
      - maltose: (+), sorbitol: —
        - arabinose: + *Sh. flexneri* type 5
        - arabinose: (+) *Sh. flexneri* type 1
      - maltose: —, sorbitol: + Serotype 425
      - maltose: —, sorbitol: — Serotype 703
  - arabinose: —
    - rhamnose: +/(+)
      - maltose: +/—, sorbitol: + *Sh. flexneri* type 3
      - maltose: (+), sorbitol: +/— *Sh. flexneri* type 4
        *Sh. boydii* type 9
    - rhamnose: —
      - maltose: +/—, sorbitol: + *Sh. flexneri* type 3
      - maltose: (+), sorbitol: — *Sh. flexneri* type 5

mannitol: +, idole: —

- xylose: +/(+)
  - arabinose: +
    - rhamnose: —
      - maltose: + , sorbitol: + Serotype 2770—51
      - maltose: (+), sorbitol: +
        - xylose: + *Sh. boydii* type 1

xylose: (+) *Sh. boydii* type 8
maltose: —, sorbitol: +
xylose: +,
dulcitol: + *Sh. boydii* type 3
xylose: (+),
dulcitol: (+) *Sh. boydii* type 6
arabinose: —
rhamnose: —
maltose: (+), sorbitol: + *Sh. boydii* type 10
xylose: —
arabinose: +
rhamnose: +
maltose: +, sorbitol: —
lactose: (+) *Sh. sonnei*
rhamnose: —
maltose: +, sorbitol: — *Sh. boydii* type 2 and 4
maltose: —, sorbitol: — Serotype 123
arabinose: —
rhamnose: —
maltose: +/—, sorbitol: + *Sh. flexneri* type 6 (Boyd 88)
mannitol: —, indole: +
xylose: +
arabinose: +/(+)
rhamnose: +
maltose: +, sorbitol: — *Sh. dysenteriae* type 8
rhamnose: —
maltose: +, sorbitol: + *Sh. flexneri* type 4
xylose: —
arabinose: +
rhamnose: (+)
maltose: (+), sorbitol: — *Sh. dysenteriae* type 7
arabinose: —
rhamnose: +
maltose: +/—, sorbitol: — *Sh. dysenteriae* type 2
mannitol: —, indole: —
xylose: +/(+)
arabinose: +
rhamnose: —
maltose: (+), sorbitol: +· Serotype 2050—52

| | |
|---|---|
| maltose: (+), sorbitol: — | Serotype 58 |
| maltose: —, sorbitol: + | *Sh. boydii* type 6 |
| maltose: —, sorbitol: (+) | Serotype 3873—50 |
| xylose: — | |
| arabinose: + | |
| rhamnose: — | |
| maltose: —, sorbitol: + | *Sh. dysenteriae* type 5 |
| maltose: —, sorbitol: (+) | *Sh. dysenteriae* type 3 |
| maltose: —, sorbitol: — | *Sh. dysenteriae* type 6 |
| arabinose: — | |
| rhamnose: — | |
| maltose: —, sorbitol: — | *Sh. dysenteriae* type 1<br>*Sh. dysenteriae* type 4 |
| maltose: +/—, sorbitol: +/— | *Sh. flexneri* type 6 (New-Castle) |
| 3. mannitol: +/—, indole: — | |
| xylose: — | |
| arabinose: — | |
| rhamnose: — | |
| maltose: +/—, sorbitol: — | *Sh. flexneri* type 6 |
| mannitol: + | *Sh. flexneri* type 6 (Manchester) |
| mannitol: — | *Sh. flexneri* type 6 (New-Castle) |

As stated above deviations of the given biochemical reactions occur. The most important, described u.o. by Kauffmann and by Ewing, are:

*Shigella dysenteriae* type 2: delayed fermenting of sucrose may be encountered.

*Shigella flexneri* types 1, 2, 3, and 5: freshly isolated cultures are sucrose-negative but subcultures may split this sugar.

*Shigella flexneri* types 1, 2, 3, 4, and 6: mannitol-negative strains may be encountered.

*Shigella flexneri* type 1: maltose and, exceptionally dulcitol may be fermented.

*Shigella flexneri* types 1, 2, 3, and 4: production of indole variable and often delayed.

*Shigella flexneri* type 6: some cultures ferment rhamnose.

## TABLE 16.

Biochemical reactions of *Bacterium anitratum* (B.5.W).

| | glucose | gas | lactose | maltose | sucrose | rhamnose | arabinose | xylose | mannitol | dulcitol | sorbitol | inositol | adonitol | salicin | indole | methyl-red | Voges Proskauer | Na-citrate | urea | $H_2S$ | gelatin | KCN test | motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B. 5. W. (*B. anitratum*) | + | — | (+)/— | — | — | — | + | + | — | — | — | — | — | — | — | +/— | — | + | — | — | — | — | — |

*Shigella boydii* type 3 and 6: fermenting of mannitol variable or delayed.

### Reference.

Edwards, P. R. and Ewing, W. H. 1955. Identification of *Enterobacteriaceae.* Burgess Publ. Comp., Minneapolis.

## B.5.w. group.

The B.5.w. group, investigated by Stuart, is a homogeneous group of bacteria in the *Enterobacteriaceae.* A striking difference of the B.5.w. group with the other groups of enteric bacteria is the inability of these cultures to reduce nitrate. On the absence of this for all *Enterobacteriaceae* characteristic reaction Schaub proposed the name: *Bacterium anitratum.* Biochemical tests will give the results summarized in table 16.

### References.

Stuart, C. A., Fonnal, S. and Mc.Cann, V. 1949. Further studies on B.5.w., an anaerogenic group of *Enterobacteriaceae.* J. inf. Dis. **84, 235.**

Brooke, M. S. 1951. The occurrence of B.5.w. (*B. anitratum*) strains in Denmark. Acta. path. microb. Scand. **28,** 338.

Ferguson, W. H. and Roberts, S. F. 1950. A bacteriological and serological study of organism B.5.w. (*B. anitratum*). J. Bact. **59, 171.**

Schaub, I. G. and Hauber, F. D. 1948. A biochemical and serological study of a group of identical unidentifiable Gram negative bacilli. J. Bact. **56,** 379.

Without doubt these tables cannot be supposed to give always satisfaction in the identification of all cultures of enteric bacteria isolated in routine laboratories, because of the impossibility of including all minor variations in biochemical behaviour encountered in the investigation of a great number of strains. For this reason, as stated before, the serological typing, if possible, will mostly be of primary importance. On the other hand, in using this key, those minor variations of strains will surely not interfere with the identification of the group of bacteria concerned and it is hoped that these schemes, mainly composed of the results published by different authors, may be an aid in the determination of *Enterobacteriaceae.*

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# THE BEHAVIOUR TOWARDS ACTINOPHAGE OF MUTANTS SURVIVING ITS LYTIC ACTION

by

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## INTRODUCTION.

The occurrence in natural substrates of actinophages, or bacteriophages attacking actinomycetes, was reported by WIEBOLS and WIERINGA (38, 39) as early as 1936. The subject, however, attracted little attention until recently, when it was discovered that an actinophage for *Streptomyces griseus* could occur in the streptomycin-production plant and cause considerable damage in the industrial process. This was observed almost simultaneously and independently by several workers: SAUDEK and COLINGSWORTH (18), WOODRUFF NUNHEIMER and LEE (40), SMITH, KUHN and MIESEL (21), REILLY, HARRIS and WAKSMAN (15).

On account of its industrial importance, *Streptomyces griseus* actinophage has been intensely studied (4,17). It was soon evident that there are several phages acting upon *Streptomyces griseus* (11), differing one from the other by their host specificity (5). According to RICE (16), neither the aerial conidia, nor the submerged spores are sensitive to the actinophage, the propagation of which can be checked in the absence of calcium (12, 23).

The early workers who studied *S. griseus* actinophage observed that resistant host-strains are readily obtained and suggested that they might be lysogenic (40). According to our own observations (26), true lysogeny does not occur with this system, but a more or less stable, mixed population of sensitive and resistant micro-organisms can very well carry the phage through numerous subcultures. An example of a probably true lysogeny in a streptomycete was recently reported by SHIRLING (19,20). This author, however, was unable to obtain typical plaques and his study was hampered by the lack of a suitable sensitive indicator strain. The occurrence of truly lysogenic actinomycetes in natural substrates is however an established fact, since we were able to isolate several *Streptomyces* species producing, after numerous successions of single colony isolations, an actinophage to which they are not sensitive, but which forms typical plaques on suitable indicator strains (33).

VAN ALSTYNE, OTTO and MCCOY (22) have shown that strains of *Streptomyces griseus* resistant to the phage are nevertheless able to adsorb it. These authors also mention attempts to isolate and study other actinophages which apparently were not published but appeared as thesis (6,9) access unto which is not easy. Similar attempts were briefly reported by GILMOUR and BUTHALA (7) and by HURD and GILMOUR (10). AZAROWICZ and PLUNKETT (1) and AZAROWICZ, ERICKSEN and ARMEN (2) succeeded in isolating *Nocardia*-phages. A few reports on actinophages also appeared in the russian literature (3, 13, 14).

In view of the peculiar morphology and life-cycle of actinomycetes, we thought that a study of their bacterial viruses, or actinophages, might be of interest to general virology.

A technique for the isolation of actinophages from natural substrates was devised (37) and proved quite successful (31). Among the many actinophages thus isolated, one acting upon the actinomycin producing strains of our collection (24, 25), actinophage $\varphi$-17, was more extensively studied (27–30, 35, 36) more especially as to its host specificity (32). In the present paper, we report the behaviour, with respect to actinophage $\varphi$-17, of isolates from *Streptomyces* S-17 that were able to withstand its lytic action.

## MATERIAL AND TECHNIQUES.

The isolation of actinophage $\varphi$-17 (32) and the properties of its host, *Streptomyces* S-17 (24, 25) were described previously.

All phage titrations (plaque count) and bacterial counts are made on nutrient-agar by the double layer method of GRATIA (8), as fully described by us (34).

Unless otherwise stated, all cultures were made in nutrient broth (peptone, meat extract, $K_2HPO_4$, NaCl) in shake flasks at 27°C.

Suspensions of conidia were obtained from slants or Roux bottles containing either peptone-asparagine agar or keratose-casein agar.

## EXPERIMENTAL.

**1.** A series of 100 ml Erlenmeyer flasks, each containing 8 ml of broth, were inoculated with 1 ml from a 24 hour-old shake-flask culture of *Streptomyces* S-17. The initial concentration of viable elements, determined by colony count, was 6 × $10^5$ B/ml. Each flask of the series received then either 1 ml of undiluted $\varphi$-17 preparation, the titer of which was 5.43 × $10^{10}$ $\varphi$/ml, or 1 ml of a range of ten fold dilutions. After incubation on the shaker at 27° for 24 hours, dilutions of the different cultures were plated for colony count and, after centrifugation, dilutions of the supernatants were plated with spores from *Streptomyces* S-17 for plaque count. The results are shown in table 1.

TABLE 1.

Influence of initial phage titer on $\varphi$-17 production.

| Culture Nr. | Initial ratio $\varphi_i/B_i$ | Phage titer after 24 h. $\varphi_f$/ml (× $10^8$) | Index of phage multiplication $\varphi_f/\varphi_i$ | Colony count, B/ml, (24 h. culture) at dilution | | |
|---|---|---|---|---|---|---|
| | | | | $10^{-1}$ | $10^{-2}$ | $10^{-4}$ |
| 1 | $10^4$ | 9 | 0.18 | 67 | 35 | 7 |
| 2 | $10^3$ | 18 | 3.6 | 19 | 8 | 0 |
| 3 | $10^2$ | 66 | 124 | 30 | 7 | 0 |
| 4 | $10^1$ | 52 | 1040 | 24 | 8 | 4 |
| 5 | $10^0$ | 26 | 5020 | 2 | 10 | 0 |

It can be seen that the final phage titer is of the same order of magnitude for all five cultures. From the ratios: final phage titer/initial phage titer, one can see that when the initial phage concentration is too high (10.000 phages for one bacterium) there is an actual loss of phage. Phage multiplication remains very low when the initial ratio is 1000 phage particles for one viable element and steadily increases as the initial ratio of phages to bacteria approaches unity.

Surviving bacteria which are able to develop into colonies are found in the 24 hour-old lysed cultures. But their number does not correlate with the dilution factor. In fact, a large part of the number of micro-organisms which are able to survive when plated at a high dilution, are unable to do so when plated at a lower dilution. Since plating dilutions of the whole culture means diluting and plating actinophage as well as bacteria, this observation means that most of the bacteria present in the lysate are sensitive to the phage: their survival on the plates is possible only if sufficient dilution gives them a chance to escape phage infection.

2. In the next experiment yeast hydrolysate was used as a culture medium and a spore suspension of *Streptomyces* S-17 as inoculum. The initial concentration of viable elements was $9.5 \times 10^5$ B/ml, the initial phage concentrations respectively: $17 \times 10^5$ and $17 \times 10^2$ $\varphi$/ml. The cultures were shaken at 27° for 2 days, and left standing at laboratory temperature for an additional ten days.

While control cultures without phage showed a good typical growth, the phage treated cultures presented after 24 hours a turbid appearance, resembling more that of *Eubacteria* than of *Actinomycetes*. A few typical granules however were seen and the microscopical examination confirmed the absence of contamination. Instead of the numerous microcolonies of *Streptomyces* such as occurred in the control, among smaller and fewer colonies a large number of branched filaments of various length could be noted. After 48 hours the picture was nearly the same. After another 10 days of incubation, all cultures, phage treated as well as control, had developed an aerial mycelium with the typical chalky layer of conidia.

Samples removed after 1 and 2 days for phage assay showed (table 2) the final titer to be of the same order of magnitude for both cultures, involving that the phage yield is higher when the initial ratio $\varphi_i/B_i$ is lower than unity. Phage assay after 12 days indicated the fairly good stability of $\varphi$-17 in the culture.

TABLE 2.

Influence of initial titer and length of incubation on $\varphi$-17 production.

| Culture Nr. | Initial ratio $\varphi_i/B_i$ ($\times$ 1.7) | Phage titer after | | | Index of multiplication | | | Colony count B/ml (12 days) at dilution | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 day | 2 days | 12 days | 1 day | 2 days | 12 days | | | |
| | | $\varphi_t$/ml ($\times$ $10^8$) | | | $\varphi_t/\varphi_i$ ($\times$ $10^3$) | | | $10^0$ | $10^{-2}$ | $10^{-4}$ |
| 1 | $10^0$ | 23 | 32 | 10 | 1.35 | 1.88 | 0.59 | 652 | 88 | 3 |
| 2 | $10^{-3}$ | 59 | 69 | 11 | 3500 | 4000 | 650 | 236 | 28 | 4 |

After thorough mixing, colony counts were made from increasing dilutions of the 12-day cultures. Again, the numbers found do not in any way correlate with the dilution factor. Since a large part of the germs actually plated in this experiment are conidia, which occurred in the liquid culture when the phage titer was already at its highest, it means that *Streptomyces* S-17, grew and sporulated in the presence of phage while keeping its original sensitivity. In fact, most of the colonies appearing on the plates were phage-contaminated, as was shown by their partial or complete lysis.

The majority of them had a very unusual appearance. They were circular, large, very flat, translucid and soft. They did not produce actinomycin. They disappeared within a few days. Attempts to obtain subcultures from this type of colony were unsuccessful, but actinophage was easily recovered from their site on the plate.

Other colonies are small and of irregular shape, of a deep yellow or orange colour, raised over the culture medium, often showing a crateriform central depression. They are soft and easily dispersed in fluid giving a turbid homogeneous suspension. They never show the typical chalky layer of aerial mycelium and conidia. Colonies of this type are also generally phage contaminated. When streaked upon nutrient agar, they develop a continuous growth layer with typical plaques. Isolation procedures show that they contain a large number of non viable organisms which form small abortive colonies later undergoing complete lysis. A few organisms however develop into healthy-looking colonies of the same type as those from which they are issued. When submitted in turn to isolation, they may prove to contain phage infected individuals, but phage-free isolates can be obtained and thereafter indefinitely subcultured. Some are sensitive to $\varphi$-17, others show some variable degree of resistance, as will be shown later.

A third type of colony which more closely resembles the normal type is also found although less frequently. These are round, often smaller than normal ones, but with the typical hard horny texture of streptomycetes. They may show the characteristic formation of aerial spores, but the chalk-white layer is often irregularly distributed, sometimes in patches, often in a very much raised circular margin. Submitted to isolation procedures, they behave exactly as colonies of the previous type, giving: non viable colonies, viable but phage contaminated colonies, and lastly phage free, either fully sensitive or more or less resistant colonies.

In no case was it possible to isolate a truly lysogenic clone, all members of which proving to be phage resistant but able to produce phage. Wherever phage was found, either in surviving colonies on the primary plates or in their subcultures after one or several isolations, the bacterial population proved to be mostly or entirely made up of phage sensitive organisms.

From these experiments as well as from previous observations made on *S. griseus* (26), it may be concluded that actinophages and sensitive streptomycetes can be propagated together in a more or less stable symbiotic equilibrium, more especially in liquid culture-media, but to some extent even on nutrient agar. This fact might be related to the low rate of actinophage fixation observed in our culture media (29).

3. The following experiments emphasize this further. Erlenmeyer flasks containing 8 ml of broth were inoculated with 1 ml of a 24-hour shake culture of *Streptomyces* S-17 and 1 ml of an increasing range of one hundred-fold dilutions of $\varphi$-17. After incubation on the shaker at 27° for 48 hours, the very dense cultures, in fact of a density similar to that of the control without phage, were centrifugated. The supernatants were assayed for phage. The bacterial sediments were re-suspended in 10 ml of broth and 1 ml of each suspension was introduced in 9 ml fresh broth to start a second passage culture. After an incubation of 48 hours, the three subcultures initially richest in phage showed an *Eubacteria*-like turbidity, while the poorest in phage, like the control one, showed a typical streptomycete-like type of growth. Samples were removed for phage assay and colony count and a third passage was started by introducing 1 ml from each culture in 9 ml of fresh broth. The procedure was repeated until the 16th passage, but not all of them were titrated. Results are shown in table 3.

Culture Nr 4, with an initial phage concentration of only 1.6 $\varphi$/ml behaves like a normal culture: no phage is recovered and colony counts agree reasonably with dilution. However, on plates used for colony count at low dilution ($10^{-2}$) from passage Nr. 2, among a crowd of normal-looking ones a few phage infected colonies could be detected. They were not observed in later passages, a fact which suggests that the culture got rid of the infecting phage through the dilution procedure involved by each subculturing. This culture remained fully sensitive.

In cultures Nr. 1, 2 and 3, where the initial phage titer was

TABLE 3.

Serial propagation of $\varphi$-17.

| Nr. | Initial phage titer $\varphi_i$/ml | Phage titer after serial passage Nr. $\varphi_t$/ml ($\times 10^8$) | | | | | Colony counts (B/ml) at dilution $10^{-6}$ after passage Nr. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 5 | 7 | 16 | 2 | 7 | 15 |
| 1 | $16 \times 10^5$ | 21 | 5.3 | 6.9 | 2.07 | 8.6 | 13 | 32 | 63 |
| 2 | $16 \times 10^3$ | 37 | 3.2 | 8.1 | 8.4 | 0.0029 | 20 | 6 | (12) |
| 3 | $16 \times 10^1$ | 0.43 | 14 | 1.65 | 4.5 | (86) | 3 | 5 | (8) |
| 4 | $16 \times 10^{-1}$ | (0) | (0) | (0) | (0) | — | (2) | (7) | — |
| | | Figures between parentheses were recorded after plating undiluted culture supernatant | | | | | Figures between parentheses indicate that correlation, with respect to dilution, was observed | | |

higher, virus multiplication occurred at first passage, although in culture Nr. 3 to a lesser extent. In the later passages it was however in the three cultures of the same order of magnitude, at least up to the 7th one. It is to be noted that colony counts made with these cultures, at high dilution, gave viable counts of the same order of magnitude as culture 4, apparently phage free, and as a control culture without phage. The main differences were that, in the phage propagating cultures, the number of colonies found on titration plates at various dilutions did not correlate with the dilution factor, and further, that most colonies were of the abnormal types described above.

Serial blind passages of cultures Nr. 1, 2 and 3 were carried until the 16th one. At that time, culture Nr. 1 was still reproducing the phage at a high concentration, but cultures 2 and 3 gave much smaller yields, their colony counts proving to correlate with the dilution factor. However, abnormal colonies were numerous and plating of these cultures, either undiluted or at low dilution, resulted in a continuous layer of growth with many typical plaques, which underwent complete lysis within a few days.

These observations show conclusively that actinophage $\varphi$-17 can be carried through several subcultures of *Streptomyces* S-17. They also show thas the sensitive streptomycete is able to multiply in broth despite the presence of a high phage concentration.

One would be tempted to interprete these observations by assuming that a true lysogeny of the *Streptomyces* occurs in broth culture, whereby the phage would be reduced to the state of prophage, and that transfer on nutrient agar would result in spontaneous induction with a high frequency. The very high titer of phage found in the broth cultures however is not in good agreement with this hypothesis and further observations made on truly lysogenic naturally occurring *Streptomyces* species (33) show these to behave quite differently.

**4.** In the following experiment, phage treated cultures were studied as to the occurrence of viable germs, able to regenerate the phage. From the 48-hour old passage Nr. 3 of culture Nr. 1, a series of ten fold dilutions were made in fresh broth. After incubation for 48 hours, a considerable increase of phage titer was observed in all subcultures containing initially at least 10 B/ml (table 4). Colony counts made for two of these subcultures also indicate that bacterial reproduction occurred, the usual titer of about $10 \times 10^6$ B/ml being obtained. Again a lack of correlation occurring between number of colonies and dilution, confirms that most of the germs remained phage sensitive.

TABLE 4.

Propagation of $\varphi$-17 from dilutions of infected cultures.

| Initial phage titer $\varphi_i$/ml ($\times$ 1.16) | Phage titer $\varphi_t$/ml ($\times 10^8$) | Colony counts (B/ml) at dilutions: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| $10^7$ | 15 | — | — | — | — | — | — | — |
| $10^6$ | 2.3 | — | — | — | — | — | — | — |
| $10^5$ | 2.7 | 85 | 76 | 38 | 150 | 270 | 53 | 9 |
| $10^4$ | 1.07 | — | — | — | — | — | — | — |
| $10^3$ | 0.21 | ($10^3$) | ($10^3$) | 526 | ($10^3$) | 412 | 74 | 8 |
| $10^2$ | 0.15 | — | — | — | — | — | — | — |
| $10^1$ | (10) | — | — | — | — | — | — | — |
| $10^0$ | (1) | — | — | — | — | — | — | — |
| Figures between parentheses were recorded with undiluted culture supernatant | | Figures between parentheses are estimates | | | | | | |

**5.** From the plates used for the colony counts of the previous experiments a number of colonies were isolated and studied.

A total of 46 isolations were performed: in 13 cases, some evidence of growth appeared along the streak and then disappeared completely. In five others, the denser parts of the streak showed a number of plaques: evidence of sensitivity and phage carrying. In the remaining 28 cases, either normal or abnormal, but healthy-looking colonies were obtained. Twenty of these were submitted to a second isolation. In 8 cases, the denser part of the streak either underwent massive lysis or showed discrete plaques, while healthy-looking colonies were isolated along the more diluted part of the streaks. In two cases, isolation revealed the presence of two different morphological variants, and in a third one even three variants were noted. This brought the total number of isolated clones under observation to 24.

They were inoculated in broth and incubated at 27° on the shaker. Eight of them refused to grow. After plating without dilution, 12 among the 16 positive broth cultures gave a continuous layer of growth, while 4 gave a growth, spotted with a large number of plaques, which lysed completely within a few days. After centrifugation, the supernatant of these four cultures contained phage producing discrete plaques on S-17 at a dilution of $10^{-8}$ in two cases, of $10^{-6}$ in two others. After 48 hours, subcultures in broth had about the same phage titer. On plates made for viable germs count, colonies of healthy appearance were formed. Five of them were isolated and inoculated in broth. After incubation no more phage was found.

The 12 broth cultures apparently phage free were plated with $\varphi$-17 at a dilution giving about 700 plaques on S-17. No plaques were formed. The experiment was repeated with a concentrated preparation of $\varphi$-17: plaques appeared on 8 of the 12 isolates tested.

The supernatants of the 12 broth cultures were plated with spores of S-17: no plaques were formed.

The 12-phage free isolates already tested as broth cultures, as well as the 8 isolates which refused to multiply in broth, were inoculated on peptone-asparagine-agar slants. Spore suspensions were made in saline. Plated on agar, they all gave a continuous layer of growth without evidence of plaques or massive lysis. Plated with decreasing dilutions of a $\varphi$-17 preparation, the titer of which was $178 \times 10^8$ $\varphi$/ml, four only proved completely resistant. All the other responded by the formation of plaques, always less in number than on the standard host S-17, and often late in appearance and of minute size.

These experiments show that isolates from phage lysates are frequently altered morphologically. They often carry the phage and in such a case they either prove non viable and undergo lysis on the isolation plate, or can easily be freed from phage by a few successive isolations. Phage free isolates are sometimes fully resistant to $\varphi$-17, more often they respond to it in a way which quantitatively and qualitatively differs from the normal host's response.

**6.** The question was therefore raised whether these 'semi-resistant' cultures were sensitive only to particular spontaneous mutants of $\varphi$-17, present in any stock phage preparation. We know already that such preparations actually contain a mutant, $\varphi$-17/5, acting on a different species of *Streptomyces*, our strain S-5 (27). If such were the case, one would expect that passage of stock phage, at a high concentration, on the semi-resistant organisms, would lead to the selection of the mutant particles. Therefore, the 16 isolates which gave some response to $\varphi$-17 were inoculated in duplicate in 9 ml of broth. One of each couple of flasks received 1 ml of phage preparation, the titer of which was $15.6 \times 10^8$ $\varphi$/ml on S-17. After incubation on the shaker for 48 hours, samples from the phage-infected cultures were taken for assay. For each strain, three new flasks, containing 8 ml of broth, were inoculated with 1 ml from the control culture and supplied with 1 ml of one hundredfold dilutions in increasing range of the supernatant from the corresponding phage-infected primary culture. After incubation for 48 hours, samples of this second passage were taken for assay. Some of the results are shown in table 5.

Knowing that the initial phage titer, as measured on S-17, was $1.56 \times 10^8$ $\varphi$/ml, in four of the cultures a limited phage multiplication, and a net decrease in the other three can be noted. This may be due, at least partly, to the high initial phage concentration (cf. table 1).

It is also obvious that the 'efficiency of plating' of the supernatant is much better on S-17 than on the homologous strain used for phage regeneration. This might be explained by the fact that most of the particles involved in the titration of this first passage are, in fact, original $\varphi$-17 phage, left over from the heavy inoculum.

However, if the second passage is considered, when supernatant from the first one was used at various dilutions to infect the homologous cultures, there is apparently no selection of mutants better

TABLE 5.

Propagation of $\varphi$-17 in cultures of semi-resistant variants.

| Isolate Nr. | Passage Nr. | | | | |
|---|---|---|---|---|---|
| | 1 | | | 2 | |
| | phage titer on strain | | initial phage dilution | phage titer on strain | |
| | homologous $(\times 10^6)$ | S-17 $(\times 10^8)$ | | homologous $(\times 10^8)$ | S-17 $(\times 10^8)$ |
| 5 | 5.2 | 1.31 | $10^{-1}$ | 0.072 | 2.02 |
| | | | $10^{-3}$ | 0.0031 | 0,16 |
| | | | $10^{-5}$ | 0.000022 | 0.0013 |
| 8 | 87 | 12 | $10^{-1}$ | 6.2 | 17 |
| | | | $10^{-3}$ | 0.94 | 15 |
| | | | $10^{-5}$ | 0.19 | 3.25 |
| 9 | 0.18 | 0.68 | $10^{-1}$ | 0.093 | 13 |
| | | | $10^{-3}$ | 0.36 | 26 |
| | | | $10^{-5}$ | 0.024 | 8.4 |
| 10 | 0.15 | 0.28 | $10^{-1}$ | 1.4 | 32 |
| | | | $10^{-3}$ | 0.12 | 6.3 |
| | | | $10^{-5}$ | (0) | (0) |
| 16 | 48 | 23 | $10^{-1}$ | 0.56 | 238 |
| | | | $10^{-3}$ | 1.26 | 66 |
| | | | $10^{-5}$ | 0.35 | 18 |
| 18 | 140 | 46 | $10^{-1}$ | 0.49 | 133 |
| | | | $10^{-3}$ | 2.83 | 85 |
| | | | $10^{-5}$ | 0.76 | 13 |
| 19 | 27 | 38 | $10^{-1}$ | 4.47 | 401 |
| | | | $10^{-3}$ | 2.6 | 190 |
| | | | $10^{-5}$ | 0.42 | 45 |

adapted to the regenerating strain. On the contrary, everywhere the efficiency of plating is considerably higher on S-17 than on its variants. In this second passage, regeneration of the phage must have taken place on the variant as the only possible host. This means that these organisms are able to produce phage particles to which they are at least relatively unsensitive.

Similar observations were reported for the reproduction of $\varphi$-17 by several different strains of actinomycin producing *Streptomyces* species (32).

7. In the following experiment broth samples inoculated with S-17 were supplied with various amounts of $\varphi$-17 and incubated on the shaker at 27° during 72 hours. After lysis of the cultures

which occurred within 24 hours, a heavy secondary growth appeared. The cultures were centrifuged and their supernatants were assayed for phage. The residual mycelium was washed 8 times with distilled water. In the eighth washing phage was titrated. Infectious centers (i.e. free phage particles plus infected complexes) and viable germs were counted in the sediment resuspended in the original amount of broth. Results are shown in table 6.

TABLE 6.

Properties of residual mycelium surviving infection by $\varphi$-17.

| Culture Nr. | Initial phage titer $\varphi_i$/ml (× 1.86) | Phage titer after 72 h. $\varphi_t$/ml (× $10^8$) | Phage titer 8th. washing $\varphi_w$/ml (× $10^4$) | Washed residual mycelium | |
|---|---|---|---|---|---|
| | | | | Infectious centers titer $\Phi$/ml (× $10^4$) | Viable germs titer B/ml (× $10^4$) |
| 1 | $10^5$ | 104 | 1.78 | 12 | 94 |
| 2 | $10^3$ | 149 | 1.49 | 5 | 25 |
| 3 | $10^1$ | 70 | 2.50 | 7 | 162 |

Apparently it is very difficult to free the residual mycelium from phage by washings. Other experiments reported elsewhere have shown that the last fractions of the phage seem to be firmly bound to the bacteria, may be in the form of virus-host complex (30). It was also shown that washed residual mycelium, diluted and incubated in broth, regenerates the phage with a burst-size which may amount to 1000.

Plated without or at low dilution the residual mycelium either lyses almost completely or gives a continuous layer of growth with a large number of plaques.

Viable organisms in the residual mycelium give rise to colonies of the various shapes already encountered and displaying the general behaviour previously discussed. From plates of colony count for culture Nr. 2, ten variants were isolated, five of them being normal-looking streptomycetes, the five other of the soft, yellow, non-sporulating type. After incubation in broth, they yielded a supernatant in which no phage acting upon S-17 could be detected. Submitted to various dilutions of a stock $\varphi$-17 preparation (table 7), they showed, like the preceding ones, a state of semi-resistance, the number of plaques formed on them being much smaller than

TABLE 7.

Variability of response to $\varphi$-17 of semi-resistant variants.

| Assay Nr. | Phage titer (× $10^6$) on strain: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | S-17 | Soft, yellow isolates | | | | Hard, sporing isolates | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 1 | 13100 | 0.97 | 0.17 | 1.72 | 0.72 | 26 | 11 | 70 | 15 |
| 2 | 5100 | 81 | 4900 | 220 | 129 | 3.5 | 4.2 | 6.8 | 1.1 |

on S-17. After being maintained in the strain collection, on peptone-asparagine agar, for some eight months with 3 or 4 mass-transfers, these ten strains were again submitted to the same test. It was found that, although their morphological characteristics had not changed, their phage-sensitivity was notably modified. It was now somewhat lower for the normal-type isolates, but considerably higher for the non-sporulating soft-type isolates, one of which had actually regained the original sensitivity of the mother strain S-17.

These observations indicate either high instability of the clones surviving phage action, or non-homogeneity of the colonies picked up. Another series of variants had therefore to be studied taking due care to purify the strains by several successive streak-isolations on nutrient agar.

**8.** Material from a well isolated plaque of $\varphi$-17 on S-17 was scraped and suspended in broth. Ten fold dilutions were made and plated with spores of *Streptomyces* S-17. Numerous isolated plaques could be noted at $10^{-6}$ and confluent lysis at $10^{-5}$; isolated surviving colonies were observed at the lower dilutions, their numbers being respectively, 11.6, 103, 106 and 14, at dilutions $10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$ and $10^{\circ}$.

From the plate of dilution $10^{-2}$, several colonies were picked up and streaked on nutrient agar. Many of them gave a continuous layer of growth spotted with plaques and were discarded. In two cases, however, a continuous layer of growth without evidence of phage contamination appeared along the heavier part of the inoculation streak. Therefore, an isolated colony, taken from the most diluted part of the inoculation streak, was selected and submitted to the same procedure, which was repeated five times in succession. This was deemed a sufficient purification and the two clones thus isolated were designed as S-17/R1 and S-17/R2. Their supernatants, after incubation in broth, did not contain phage acting upon S-17.

Their sensitivity to various preparations of $\varphi$-17 and of its host-mutant $\varphi$-17/5 (27) was recorded on several occasions within a period of six months. The figures obtained are shown in table 8, where is also shown the activity of the phages tested on streptomycetes S-17 and S-5, the latter strain being used as an indicator for mutant particles $\varphi$-17/5 spontaneously occurring in any $\varphi$-17 stock.

It is readily seen from the percentage columns in the upper part of the table that the frequency of mutant particles acting upon S-5 is relatively constant in the different preparations tested. In conformity with our previous observations (27, 32), it varies roughly between $10^{-5}$ and $10^{-4}$. On the contrary, the frequency of particles able to form plaques on either S-17/R1 or S-17/R2 varies much more widely, from $10^{-8}$ to $10^{-3}$. There is complete parallelism for the variations of phage activity on R1 and R2, the titer being always higher on the former strain, this factor varying between 2 and 30. There is also some evidence of parallelism between phage activity on S-5 on the one hand, on R1 and R2 on the other. Although not so striking as in the former case, it suggests the possibility of a relationship between phage particles acting upon S-5 and upon the variants R1 and R2. So the sensitivity of the latter organisms to $\varphi$-17/5 needed further investigation.

The lower part of the table indicates that preparations of phage $\varphi$-17/5 have about the same activity on both S-17 and S-5. They contain particles able to form plaques on R1 and R2, with a frequency of the order of $10^{-5}$, which in one case rose up to $10^{-2}$ for R1 while unexpectedly dropping at the same time to *nil* for R2. So phage $\varphi$-17/5, isolated on S-5 and thereafter stabilized by several passages on S-17, cannot be concluded to be more active than the original $\varphi$-17 upon variants R1 and R2 and thus is no host mutant for these strains.

The activity of freshly isolated $\varphi$-17/5 was however to be examined. In this aim, plaque counts on the four indicators were performed with the material scraped from well isolated plaques made on S-5 by $\varphi$-17. For comparison, the same experiment was also performed with material scraped from plaques made by $\varphi$-17 on R1 and on R2. From the middle part of table 8, it is seen that $\varphi$-17/5, freshly isolated from a single plaque, is from 3 to 20 times more active on S-5 than on S-17. Simultaneously, the ratio of particles

TABLE 8.

Sensitivity of variants S-17/R1 and S-17/R2 to different preparations of actinophages $\varphi$-17 and $\varphi$-17/5.

| Origin of phage | | Phage titer on strain | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | S-17 | S-17/R1 | | S-17/R2 | | S-5 | |
| | | $\varphi$/ml | $\varphi$/ml | per cent | $\varphi$/ml | per cent | $\varphi$/ml | per cent |
| $\varphi$-17 | | $(\times 10^6)$ | $(\times 10^2)$ | (*) | $(\times 10^2)$ | (*) | $(\times 10^4)$ | (*) |
| S-17 | plaque | 3.75 | 1.03 | 0.0027 | 0.34 | 0.0009 | 0.03 | 0.008 |
| S-17 | broth | 2300 | 120 | 0.0005 | 11 | 0.00005 | 7.3 | 0.003 |
| S-17 | broth | 2600 | 4.6 | 0.00002 | 0.18 | 0.0000007 | 1.89 | 0.0007 |
| S-17 | broth | 3200 | 0.42 | 0.0000013 | 0.23 | 0.0000007 | 2.8 | 0.0009 |
| S-17(1) | broth | 5100 | 49000 | 0.096 | 3200 | 0.0063 | 36 | 0.0071 |
| S-17(2) | broth | 51000 | 370 | 0.000073 | 211 | 0.000041 | 98 | 0.0019 |
| S-5 | plaque | 0.14 | 105 | 7.5 | 73 | 5.2 | 47 | 335 |
| S-5 | plaque | 0.16 | 320 | 20 | 341 | 21 | 350 | 2200 |
| S-17/R1 | plaque | 0.006 | 810 | 1350 | 207 | 345 | 7.2 | 1200 |
| S-17/R1 | plaque | 0.023 | 312 | 135 | 304 | 132 | 6.1 | 265 |
| S-17/R2 | plaque | 0.023 | 2700 | 1170 | 1200 | 520 | 11 | 480 |
| S-17/R2 | plaque | 0.082 | 15700 | 1915 | 6300 | 770 | 121 | 1475 |
| $\varphi$-17/5 | | $(\times 10^8)$ | $(\times 10^4)$ | | $(\times 10^4)$ | | $(\times 10^8)$ | |
| S-17 | broth | 24 | 21 | 0.0087 | 6.5 | 0.0027 | 97 | 405 |
| S-17 | broth | 12 | 2500 | 2.08 | 0 | — | 17 | 142 |
| S-17 | broth | 9.2 | 6.4 | 0.007 | 2.47 | 0.0027 | 6.5 | 71 |

(1) Assay made several months after the other ones, the microorganisms were kept on peptone-asparagine agar at 2°—4° and were submitted to 4 subcultures.

(2) Large scale production in 150 liters tanks.

(*) The corresponding titer on S-17 being taken as 100 per cent.

forming plaques on R1 and R2 rises to 5 or 20 per cent, the two variants giving a similar response. Also the phage present in a single isolated plaque formed by φ-17 on either R1 or R2 is definitely more active on S-5, R1 and R2 than ordinary φ-17 regenerated on S-17. In fact, the titer is of the same order of magnitude on each of these three strains, and constantly higher, by a factor reaching up to 20, than on *Streptomyces* S-17.

It therefore appears that, contrary to observations made with other variants (table 5), there is a selection of better adapted phage particles by passage on strains R1 and R2. Further, the phage acting upon these organisms might be identical with φ -17/5.

**9.** Serial passage of φ-17 on strains S-17/R1 and S-17/R2 was therefore undertaken.

The material scraped from an isolated plaque formed on R1 by φ-17 was taken up in broth, diluted and plated with each of the four indicator strains. The procedure was repeated with the material from a plaque of this second isolation on R1, and again, up to 9 passages.

It can be seen (table 9) that the phage yield of the plaques selected, tested on R1, is always low and shows wide variations. But the titer of the plaque material is of the same order of magnitude whether assayed on R1 or on any one of the three other strains, R2, S-17 or S-5.

Material from the eighth plaque-passage was introduced into a broth culture of R1, the supernatant of which, after incubation, was passed into a second culture, and so on, four times in succession (passages 9a to 12a). A constant drop of phage titer was observed, indicating that S-17/R1 does not act as a good selector of mutant phage particles, such as S-5 in the case of φ-17/5 (27).

The supernatant from the first broth passage on R1 (passage 9a), was introduced into broth cultures of S-17 (passages 10b to 13b), R2 (passages 10c to 12c) and S-5 (passages 10d to 13d) and serially passed three or four times on the corresponding microorganism. A rapid drop of phage yield was observed with R2, as it had been the case with R1. Moderate yield was maintained with S-5, but, when the phage was subsequently passed on either R1 (passage 13d/1) or R2 (passage 13d/2), a sharp drop in phage production occurred. During passage on S-17, a progressive increase of phage-yield was observed, accompanied by a much higher relative in-

TABLE 9.

Serial propagation of $\varphi$-17 on S-17/R1.

| Passage Nr. | On strain | Method | Phage titer on strain | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-17/R1 | S-17/R2 | | S-17 | | S-5 | |
| | | | $\varphi$/ml ($\times 10^2$) | $\varphi$/ml ($\times 10^2$) | per cent (*) | $\varphi$/ml ($\times 10^2$) | per cent (*) | $\varphi$/ml ($\times 10^2$) | per cent (*) |
| 1 | R1 | pl | 0 | 0 | — | 0 | — | 120 | — |
| 2 | R1 | pl | 0.22 | 0.03 | 13.6 | 0.28 | 127 | 0.37 | 168 |
| 3 | R1 | pl | 74 | 98 | 132 | 31 | 42 | 38 | 51 |
| 4 | R1 | pl | 37 | 157 | 425 | 25 | 68 | 94 | 270 |
| 5 | R1 | pl | 0.54 | 0.28 | 52 | 0.23 | 42.5 | 0.09 | 16.7 |
| 6 | R1 | pl | 0.09 | 0.03 | 33.3 | 0.11 | 122 | 0.25 | 278 |
| 7 | R1 | pl | 0.14 | 0.03 | 21.4 | 0.01 | 7.15 | 0.15 | 10.7 |
| 8 | R1 | pl | 3.6 | 1.16 | 32.2 | 3.2 | 89 | 3.5 | 97 |
| 9 | R1 | pl | 0.27 | 0.25 | 92.5 | 0.15 | 55.5 | 1.09 | 405 |
| 9a | R1 | br | 14 | 16 | 114 | 1.12 | 8 | 38 | 270 |
| 10a | R1 | br | 8.1 | 2.4 | 29.8 | 1.26 | 15.5 | 0.28 | 3.5 |
| 11a | R1 | br | 0.75 | — | — | — | — | — | — |
| 12a | R1 | br | 0.03 | — | — | — | — | — | — |
| 10b | 17 | br | 34 | 36 | 106 | 8 | 23.5 | 30 | 88 |
| 11b | 17 | br | — | — | — | 1.32 | — | — | — |
| 12b | 17 | br | — | — | — | 140 | — | — | — |
| 13b | 17 | br | $77 \times 10^4$ | $20 \times 10^4$ | 26 | $43 \times 10^6$ | 5600 | $32 \times 10^4$ | 41.5 |
| 13b/1 | R1 | br | — | — | — | 26 | — | — | — |
| 13b/2 | R2 | br | — | — | — | 18 | — | — | — |
| 10c | R2 | br | 20 | 2.09 | 10.4 | 1.38 | 6.9 | 0.9 | 4.5 |
| 11c | R2 | br | 0.45 | — | — | — | — | — | — |
| 12c | R2 | br | 0.01 | — | — | — | — | — | — |
| 10d | 5 | br | 1700 | 550 | 32.4 | 153 | 9 | 470 | 27.6 |
| 11d | 5 | br | — | — | — | — | — | 6 | — |
| 12d | 5 | br | — | — | — | — | — | 2.53 | — |
| 13d | 5 | br | — | — | — | — | — | 182 | — |
| 13d/1 | R1 | br | — | — | — | — | — | 0.06 | — |
| 13d/2 | R2 | br | — | — | — | — | — | 0.07 | — |

(*) The corresponding titer on S-17/R1 being taken as 100 per cent.

crease of activity upon *Streptomyces* S-17. A reverting to strain R1 (passage 13b/1) or R2 (passage 13b/2) as host produced again an immediate and sharp drop of phage production.

It can be concluded that strain S-17/R1, a variant of S-17 surviving the lytic action of phage $\varphi$-17, displays a sensitivity of a low order to this phage. It allows to a small extent its multiplication the phage thus formed having, contrary to the original one, the same order of activity upon each of the four indicators. This is not a stable mutant of $\varphi$-17, as is $\varphi$-17/5, since return to the normal host S-17, after several serial passages on R1, results immediately in a higher yield with quick reappearance of a phage possessing the host specificity of the original one.

**10.** Similar but less extensive passage experiments were performed on *Streptomyces* S-17/R2. The results are presented in table 10.

TABLE 10.

Serial propagation of $\varphi$-17 on S-17/R2.

| Passage Nr. | On strain | Method | Phage titer on strain: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-17/R2 | S-17/R1 | | S-17 | | S-5 | |
| | | | $\varphi$/ml ($\times 10^4$) | $\varphi$/ml ($\times 10^4$) | per cent (*) | $\varphi$/ml ($\times 10^4$) | per cent (*) | $\varphi$/ml ($\times 10^4$) | per cent (*) |
| 1 | R2 | pl | 0.25 | 0.55 | 220 | 0.89 | 356 | 0.52 | 208 |
| 2 | R2 | pl | 30 | 60 | 200 | 150 | 500 | 11 | 366 |
| 3 | R2 | pl | 0.08 | 0.25 | 313 | 0.50 | 625 | 0.21 | 262 |
| 4 | R2 | pl | 0.03 | 0.11 | 367 | 0.31 | 1000 | 0.12 | 400 |
| 5 | R2 | pl | 0.34 | 0.93 | 274 | 0.83 | 244 | 0.49 | 144 |
| 6 | R2 | pl | 0.64 | 2.3 | 360 | 12 | 1875 | 7.3 | 1140 |
| 7 | R2 | pl | 0.025 | 0.25 | 1000 | 0.34 | 1360 | 0.35 | 1400 |
| | | | ($\times 10^6$) | ($\times 10^6$) | | ($\times 10^6$) | | ($\times 10^6$) | |
| 8 | R2 | br | 8.1 | 40 | 495 | 0.95 | 11.7 | 42 | 520 |
| 9 | R2 | br | 5.6 | 7.2 | 128 | 8.3 | 148 | 8.6 | 153 |
| 9a | 17 | br | 16 | 2.7 | 16.9 | 12 | 75 | 17.8 | 111 |
| 9b | 5 | br | 2.77 | 3.14 | 115 | 4.23 | 153 | 3.22 | 117 |
| 9c | R1 | br | 5.8 | 5.3 | 91 | 5.6 | 96 | 1.34 | 23 |

(*) The corresponding titer on S-17/R2 being taken as 100 per cent.

Passage of material scraped from isolated plaques (passages 1 to 7) as well as broth passages (passages 8 and 9) give a much higher yield (plaque count) than with R1. The activity of the phage thus obtained is of the same order of magnitude on each of the four strains tested. It is however generally somewhat higher, the factor varying from 2 to 18, on R1, S-17 and S-5, although the regeneration took place on R2.

Thus it is made obvious that the behaviour towards actinophage of bacterial mutants surviving the lytic action of the latter may vary greatly.

## Conclusions and summary.

1. Within a wide range of initial actinophage $\varphi$-17 concentrations, the final titer of broth cultures, inoculated with either young mycelium or conidia of *Streptomyces* S-17, remains of the same order of magnitude.

2. Phage lysates of this organism sometimes become perfectly clear; but, more often, they show a turbid appearance resembling that of cultures from typical *Eubacteria*.

3. Quite generally, lysis is followed by the development of a secondary growth, in the shape of residual mycelium if the culture is kept on the shaker, in the form of aerial mycelium and conidia if at rest.

4. In either case, most of the elements constituting this secondary growth retain the phage-sensitivity of the original culture, despite the fact that they arose in a medium already very rich in actinophages.

5. Through repeated transfers of this secondary growth in fresh culture medium, actinophage and streptomycete can be serially propagated together through many subcultures, most of the bacterial population keeping its original sensitivity to the virus.

6. Isolated colonies formed on nutrient agar from the secondary growth surviving phage action may have a normal appearance, but most of them are abnormal variants. Most conspicuous among them are: translucid, colourless, flat colonies; raised, deep yellow or orange, soft, non-sporulating colonies; hard, horny colonies on which aerial mycelium and conidia appear in irregular patches. Those same types are also found when a heavy spore suspension of the streptomycete is plated with a low dilution of phage.

7. All types of colonies are liable to show evidence of phage-contamination, either by their complete and massive lysis after a period of abortive growth or by partial lysis, in sector or of the moth-eaten type.

8. When submitted to streak isolation, on nutrient agar, isolated colonies surviving phage action, normal or abnormal, but showing no evidence of phage infection, may nevertheless prove to carry the virus. A second and possibly a third isolation are then sufficient to eliminate the phage and obtain virus free isolates.

9. True lysogeny was not found in any of the many isolates studied.

10. Phage free isolates are often as sensitive to the phage as the parent organism. Rarely, they prove completely resistant to its action, a concentrated phage preparation not forming any plaque when plated with their spore suspensions. More often, they show a state of 'semi-resistance', a concentrated phage preparation, plated with their spore suspensions, forming a number of plaques, but much below the number on its normal host S-17.

11. The behaviour towards actinophage $\varphi$-17 of these semi-resistant strains is variable.

12. In some of the cases studied, the variants regenerate the phage with a good efficiency, but a large proportion of the particles thus synthesized, are unable to form plaques on the homologous strain itself and are detected only by the use of the parent organism S-17 as indicator.

13. In other cases, the semi-resistant variant regenerates the phage with a very low or a moderately good efficiency, but the particles thus synthesized differ from the parent phage by their host specificity. They are able to form plaques on the semi-resistant variants as well as on the original *Streptomyces* S-17 and may be considered as host mutants.

14. When such is the case, the phage formed by the semi-resistant variant also acts upon *Streptomyces* S-5, a strain belonging to a species different from S-17, by means of which a stable spontaneous host mutant of $\varphi$-17 (called $\varphi$-17/5) was isolated previously.

15. The actinophage regenerated by the semi-resistant variants of S-17 is not identical with $\varphi$-17/5. Contrary to this virus, it is quickly replaced by the original $\varphi$-17 after passage on S-17. Besides, $\varphi$-17/5, stabilized by several passages on S-17, forms on these variants only a small part of the number of plaques which it gives on either S-17 or S-5.

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# SOME OBSERVATIONS ON PROPIONIC ACID FERMENTATION IN SILAGE

by

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(Received October 20, 1956).

## INTRODUCTION.

Using chromatographic techniques (BROUWER and NIJKAMP (1950), VIRTANEN and MIETTINEN (1951) and BARNETT and DUNCAN (1954) demonstrated the presence of propionic acid in several samples of silage.

According to LEPPER (1938) other volatile acids than acetic and butyric acids are only present in silages which contain much butyric acid. This would justify his method for determining the amount of butyric acid in silage by distilling an extract of the silage and titrating only two fractions; from the titration figures the amounts of acetic and butyric acids in the silage are computed. Only for silage containing much butyric acid the method would be defective. The results obtained by BROUWER and NIJKAMP (1950), VIRTANEN and MIETTINEN (1951) and BARNETT and DUNCAN (1954) seem to bear out LEPPER's statements.

Propionic acid could be formed in silage by normal propionic acid bacteria, but for instance also by bacteria like *Micrococcus lactilyticus* (FOUBERT and DOUGLAS, 1948), *Lactobacillus bifermentans* (PETTE and VAN BEYNUM, 1943), *Clostridium propionicum* (CARDON and BARKER, 1947). This last mentioned bacterium was indeed isolated from grass by JOHNS (1952).

Some observations on propionic acid fermentation in potato pulp silages and in silages of grass mixed with potato pulp are now presented.

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## METHODS.

Volatile acids were determined either by chromatography (HISCOX and BERRIDGE, 1950) or, together with lactic acid, by the distillation method of LEPPER-FLIEG (see HERRMANN, 1941). No steam distillation was used before chromatography (VIRTANEN and MIETTINEN, 1951).

Cultures of propionic acid bacteria were identified according to the system of VAN NIEL (BERGEY, 1948).

Propionic acid bacteria were isolated by shake cultures in a medium containing 1% yeast extract (Difco), 0.5% Bactotryptone, 1.5% sodium lactate and 2% purified agar; pH 6.5. From a colony in the third subsequent shake culture the bacteria were finally plated on the same medium. A well isolated colony obtained after incubating anaerobically was accepted as a pure culture. The pure cultures were kept as stab cultures in lactate agar.

The fermentation of different substrates was investigated in a medium containing 0.5% yeast extract (Difco) to which 1% of substrate was added as a 10% solution in water. Solutions of yeast extract and substrate were sterilised separately. The solutions of pectic acid, arabinose, xylose, mannose, and maltose were sterilised by filtration, the other solutions by heat. Tubes with 10 ml of medium were inoculated with one drop of the culture investigated and incubated anaerobically for fourteen days at 30°C. Then the contents of the tubes were titrated to the pH of an uninoculated blank, about 6.0. The inoculum was obtained from a young anaerobic culture in a medium containing 1% of glucose and 0.5% of yeast extract.

This same medium was used for investigating gas and propionic acid production from glucose, acetoin and diacetyl formation (Voges-Proskauer test) and nitrate reduction; in the last case the medium was supplemented with 0.5% of potassium nitrate. Gas production was checked in evacuated tubes (VAN BEYNUM and PETTE, 1942). Qualitative tests for nitrite and nitrate were performed according to Manual of Methods (1954).

Catalase was looked for in colonies grown anaerobically on lactate yeast extract agar. Bacteria from this medium were also used for the Gram stain.

Motility was checked in hanging drops of very young cultures in media with 0.5% yeast extract and either glucose (1%) or lactate (1%).

## RESULTS.

### Propionic acid in potato pulp.

The press juice from ten samples of potato pulp of different origins was analysed chromatographically. It was found that seven samples contained propionic acid. As, however, these samples were very old, about 3 years, too much importance could not be attached to these observations. Therefore next 21 samples were analysed which were only a few months old. In 11 of them the presence of propionic acid could again be demonstrated, but this time only a trace (maximally about 0.03%).

In samples of potato pulp of abnormal composition, also containing more protein then usual, more propionic acid was found, however. See table 1 which also gives the amount of butyric acid found.

TABLE 1.

The amount of butyric and propionic acids in samples of potato pulp of deviating composition.

| sample No. | propionic acid | butyric acid |
|---|---|---|
| 1 | 0.07 | 0.11 |
| 2 | 0.03 | —[1] |
| 3 | 0.09 | 0.14 |
| 4 | 0.09 | 0.12 |
| 5 | 0.09 | — |
| 6 | 0.07 | >0.22 |
| 7 | >0.18 | ≫0.22 |
| 8 | 0.09 | 0.16 |

[1] means that the acid could not be detected.

### Propionic acid in silages of grass mixed with potato pulp.

In table 2 results are given of determinations of acids with the distillation method and for butyric acid also the chromatographic method. In all cases less butyric acid was found chromatographically. This was caused by the presence in all samples of considerable amounts of propionic acid, as shown by large spots on the chromatograms.

TABLE 2.

Comparison of distillation and chromatographic methods for the determination of butyric acid.

| silage No. | pH | Amount of acid determined by distillation according to LEPPER-FLIEG lactic | acetic | butyric | Amount of acid determined chromatographically according to HISCOX and BERRIDGE butyric |
|---|---|---|---|---|---|
| | | % | % | % | % |
| 1 | 4.9 | 0.51 | 0.98 | 0.59 | 0.2 [1] |
| 2 | 4.1 | 1.70 | 0.48 | 0.52 | 0.2 [1] |
| 3 | 4.0 | 1.40 | 0.86 | 0.09 | 0.04 |
| 4 | 3.8 | 2.52 | 0.59 | 0.03 | 0.00 |
| 5 | 3.8 | 2.44 | 0.81 | 0.07 | 0.00 |
| 6 | 4.1 | 0.93 | 1.25 | 0.15 | 0.02 |
| 7 | 4.6 | 0.86 | 1.17 | 0.33 | 0.1 |

[1] In all samples propionic acid was present but in these samples also valeric and capronic acids were found.

It is clear from these results that the distillation method is wholly unsuited for the determination of butyric acid in silages of mixed grass and potato pulp. It seems to us that this method should also be condemned for the determination of butyric acid in silages of grass alone, as butyric acid can never be demonstrated by it with absolute certainty. Moreover the method of HISCOX and BERRIDGE (1950) is very simple and suited for mass determinations.

**Isolation of propionic acid bacteria from potato pulp.**

From each of 6 samples of potato pulp a culture of propionic acid bacteria was isolated.

All were found to be immobile, Gram positive and catalase positive rods. The rods in some cases were very short or irregularly shaped. Aerobically growth was hardly obtained. Chromatographically it was shown that propionic acid was formed from glucose. Carbon dioxide was also formed from glucose, as sole gaseous product. It is obvious therefore that the bacteria isolated were indeed bacteria belonging to the genus *Propionibacterium* Orla-Jensen.

The growth of all strains could be described as cream coloured. A positive Voges-Proskauer reaction was obtained in all cases.

Table 3 shows that two of the cultures, nrs 1 and 2, may be identified as *Propionibacterium freudenreichii*. These cultures deviated from VAN NIEL's description mainly in secondary properties. However, it should be noted that neither of these cultures formed

TABLE 3.

Comparison of the properties of the strains of propionic acid bacteria isolated by us, with the characteristics of the known species.

| Species according to VAN NIEL (BERGEY, 1948) | Fermentation of different substrates | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | amygdalin | D(-)-arabinose | DL-arabinose | L(+)-arabinose | cellobiose | glucose | glycogen | inositol | lactose | maltose | mannitol | pectin[1] | D(+)-raffinose | rhamnose | saccharose | salicin | sorbitol | xylose | starch | catalase | nitrate reduction |
| *Propionibacterium* | | | | | | | | | | | | | | | | | | | | | |
| *freudenreichii* | —[2] | — | . | — | . | + | — | . | — | + | — | . | — | — | — | . | . | — | . | + | — |
| *shermanii* | — | . | ∓ | . | . | + | — | + | **+** | — | — | . | — | — | — | — | — | — | — | + | — |
| *rubrum* | + | — | . | — | . | + | — | . | + | **+** | **+** | . | **+** | — | **+** | + | — | — | — | + | — |
| *thoenii* | — | . | — | . | . | + | — | . | + | **+** | — | — | — | — | **+** | + | **+** | — | — | + | — |
| *zeae* | . | — | . | **+** | + | + | — | . | + | **+** | + | . | . | **+** | **+** | + | . | — | — | + | — |
| *petersonii* | . | — | . | — | — | + | — | . | + | **+** | . | — | — | — | **+** | + | — | — | — | + | — |
| *jensenii* | — | — | . | — | — | + | — | + | + | **+** | ± | — | ± | — | **+** | **—** | — | — | — | + | — |
| *raffinosaceum* | + | — | . | — | **+** | + | — | + | + | **+** | + | — | + | — | **+** | **+** | — | — | — | + | — |
| *technicum* | + | . | + | . | . | + | **+** | . | + | **+** | + | . | + | . | **+** | . | . | — | **+** | + | — |
| *arabinosum* | . | **+** | . | **+** | + | + | . | . | . | + | + | . | + | — | + | — | + | — | . | (—) | . |
| *pentosaceum* | . | **+** | . | **+** | + | + | — | + | + | + | + | — | + | **+** | + | + | + | **+** | . | (+) | **+** |
| The strains isolated by us | Amount of acid from the substrates mentioned above in ml N/10 NaOH per 10 ml of medium[3] | | | | | | | | | | | | | | | | | | | | |
| 1 | 0.0 | 0.1 | | 0.9 | 0.0 | 5.6 | 0.0 | 1.8 | 0.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.0 | + | — |
| 2 | 0.0 | 0.1 | | 0.1 | 0.1 | 3.9 | 0.0 | 1.9 | 0.1 | 0.1 | 5.6 | 0.0 | . | 0.9 | 0.2 | 0.3 | 5.0 | 0.1 | 0.1 | + | — |
| 3 | 0.2 | 0.1 | | 4.8 | 3.1 | 5.9 | 0.1 | 3.5 | 7.0 | 2.2 | 7.9 | 0.0 | 0.2 | 0.0 | 5.2 | 0.6 | 1.8 | 4.8 | 0.2 | + | — |
| 4 | 0.3 | 0.2 | | 3.7 | 2.3 | 6.9 | 0.1 | 3.2 | 4.9 | 3.5 | 7.7 | 0.1 | 0.1 | 5.2 | 2.5 | 0.3 | 2.7 | 5.5 | 0.2 | + | — |
| 5 | 0.1 | 0.1 | | 4.9 | 0.8 | 5.8 | 0.0 | 0.0 | 5.4 | 5.0 | 8.6 | 0.0 | 0.2 | 0.0 | 3.7 | 0.2 | 6.1 | 7.4 | 0.1 | + | — |
| 6 | 0.1 | 0.1 | | 5.0 | 3.1 | 5.8 | 0.0 | 3.8 | 5.0 | 4.7 | 6.1 | 0.1 | 0.2 | 7.6 | 6.7 | 0.7 | 2.5 | 6.4 | 0.2 | + | — |

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[1] In our experiments a preparation of pectic acid (Eastman, technical) was used. This preparation also contained some galactan and other polysaccharides (DE MAN, 1957).

[2] Meaning of signs: + positive, — negative, ∓ generally negative, ± generally positive, (—) nearly negative, (+) weakly positive.
Properties of primary importance in VAN NIEL's system are in bold type.

[3] Positive results are in bold types.

coccoid cells in a medium with glucose, which *Pbm freudenreichii* should.

The four remaining cultures could be identified with still less certainty than the first two. They may be regarded as strains of *Pbm zeae*. Important deviations from the description of this species occurred, however. Especially the fact that all four cultures could ferment xylose draws the attention.

It is interesting to note that the first strain of *Pbm zeae* was probably also isolated from silage (Bergey, 1948).

Propionic acid bacteria were sometimes found in potato pulp in large numbers. It seems certain therefore that the propionic acid sometimes found in potato pulp is formed by these bacteria.

## Summary.

Propionic acid was found in samples of potato pulp silage and of silage made from mixed grass and potato pulp. Often butyric acid was present besides propionic acid. This implies, as was also demonstrated, that the Lepper distillation method can not be used for the detection of butyric acid in the kinds of silage mentioned. Its use with other kinds of silage seems unsafe.

The propionic acid was probably formed by propionic acid bacteria. Six cultures of these were isolated. Two were identified as *Propionibacterium freudenreichii* and the remaining cultures tentatively, as *Pbm zeae*.

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# THE FERMENTATION OF CELL WALL SUBSTANCES IN GRASS SILAGE AND IN POTATO PULP

by

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(Received October 20, 1956).

## INTRODUCTION.

It is an accepted fact that in silage soluble carbohydrates — mainly fructose, glucose, saccharose and fructan — are transformed into acids and other products. About the fate of the 'insoluble' cell wall substances much less is known, however. During the last few years indications have been obtained that these may also be fermented.

According to ORLA-JENSEN (1947) in old silage pentosans are probably fermented by *Betabacterium pentoaceticum*.

OLSEN (1951) found that hexosans and pentosans in sugar beet pulp are transformed into acids, the hexosans at least probably by lactic acid bacteria. The significance of these results is very doubtful however as rather unspecific methods of determination were used. Hexosans were determined as the difference of dry matter and total amount of other substances.

ARCHIBALD (1953) observed that in several silages more acid was formed than was possible from the amount of sugars originally present, which also seems to point to fermentation of cell wall substances.

Using chromatographic methods WYLAM (1953) demonstrated the presence of galactose, arabinose and xylose in silage. These sugars were not present in the original silage. The fact that they

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were not fermented must no doubt be ascribed to the high soluble carbohydrate content of the ensiled grass. Probably the sugars mentioned were formed from polysaccharides, possibly by the enzymes of the grass itself.

Both ARCHIBALD (1953) and KROULIK *et al.* (1955) found a larger amount of sugar in silage, made with sulfite, than was present in in the ensiled grass. Again formation of the sugars, from cell wall substances, by plant enzymes does not seem to be excluded.

One may conclude that it seems very probable that cell wall substances may indeed be fermented in silage, but that there is hardly any evidence that lactic acid bacteria can attack these substances without the help of enzymes from the material ensiled.

During experiments on the use of potato pulp as an additive for grass silage some observations on the fermentation of cell wall substances in silage were made, which are the subject of this communication.

## METHODS.

### Preparation of silage.

Silage was made in preserving jars (SCHIEBLICH, 1930). The jars were closed with airtight rubber gaskets. The lids were kept in place with springs. If a pressure developed inside the jars, gas could escape. The jars were kept at 20°C.

### Determinations in grass and grass silage.

The amount of soluble carbohydrates in grass and grass silage was determined colorimetrically with anthrone, by a method similar to that of YEMM and WILLIS (1954).

The amount of sugar formed on hydrolysis with hydrochloric acid by the method of SACHSSE (1877) — using N/1 instead of 0.7 N acid, however — was determined with the copper-iodometric method of SCHOORL (1929).

The acids in grass silage were determined by the method of LEPPERFLIEG (see HERRMANN, 1941). The total amount of acid can undoubtedly be determined by this method, although it is not reliable for the determination of the separate volatile acids.

### Determinations in ordinary potato pulp.

The carbohydrates starch, galactan, araban and xylan in potato pulp were determined by hydrolyzing the dried potato pulp with

N 1 sulfuric acid for 2½ hours at 100°C., neutralizing with barium carbonate and determining the sugars after separation by paper chromatography on Whatman No 1 paper. The solvent used was ethyl acetate-pyridine-water (10 : 3.5 : 2.5). This solvent gives a separation of the sugars as good as that of Mc FARREN, BRAND and RUTKOWSKI (1949) (same substances, 2.5 : 1 : 3.5, upper layer), but it has the added advantage that no separation of layers is necessary in its preparation. The sugars were eluted from the paper using the technique of FLOOD, HIRST and JONES (1947) and determined colorimetrically (NELSON, 1944).

Other substances determined in potato pulp were cellulose (CRAMPTON and MAYNARD, 1938), pectic acid (Mc CREADY and Mc COMB, 1952), lactic, acetic and butyric acids (LEPPER-FLIEG, see HERRMANN, 1941), protein (Kjeldahl) and ash.

## Preparation and investigation of silage made with sterilised potato pulp.

Experiments on fermentation of potato pulp in pure culture were made by sterilizing 2 g lots of unfermented, dried potato pulp, in tubes furnished with cotton plugs, by means of ethylene oxide.

To the sterilised pulp additions were made of 20 ml of water or yeast extract solution, to which cells of *Streptobacterium casei*, obtained from agar slants, had been added if necessary.

After incubation for 2½ months at 25°C., tests for occurrence of infection were made by bringing a loopful of the contents of the different tubes in, or on, a medium containing tryptone, 1%; meat extract, 1%; yeast extract, 0.5%; tomato juice, 4%; Tween 80, 0.1%; $K_2HPO_4$ 0.2%; glucose, 2% and, if necessary, agar, 1.5%. The pH was 6.8. The tests were performed both aerobically and anaerobically at 25°C., aerobically on the solid medium and anaerobically with the liquid medium in tubes which were evacuated and heat sealed after inoculation.

Chemical determinations were made in extracts obtained by adding 6 ml of water to 2 g of pulp, titrating to the pH of an uninoculated blank and filtrating.

In 1 ml of filtrate volatile acids were determined after distillation in a MARKHAM (1942) apparatus. Before distillation 0.2 ml of 2N sulfuric acid were added. 50 ml of distillate were collected and titrated with N/100 NaOH, with phenol red as indicator.

Lactic acid was determined in 0.5 ml of extract by the method of

Long (Elsden and Gibson, 1954). The acetaldehyde formed was removed using a stream of nitrogen gas (Gerritsma, 1954).

The pulp not used for making the extract, was dried at 105°C. 1 g of the dried product was hydrolysed with 25 ml of N/1 sulfuric acid and a chromatogram of the neutralised hydrolysate was prepared as described in the preceding paragraph. Aniline phosphate in ethanol was used as spray reagent. The amounts of the sugars on the paper were estimated this time by comparing the spots with those obtained with reference solutions.

**Identification of lactobacilli.**

The medium used for determining the fermentation of glucose and melibiose contained: Bactotryptone, 2%; yeast extract, 1%; $K_2HPO_4$, 0.2%; $MgSO_4 \cdot 7aq$, 0.1%, and sugar, 2%, in distilled water. The pH was 7.0. After inoculation, tubes with this medium were incubated at 30°C. for 14 days, then titrated with N/10 alkali with phenolphthalein as indicator.

Gas production from glucose or citrate was determined in the following medium: Bactotryptone, 1%; Oxo meat extract, 1%; Difco yeast extract, 0.5%; tomato juice, 4%; Tween 80, 0.1%; $K_2HPO_4$, 0.2% and glucose, 2% or citric acid, 3% in distilled water; pH 6.8. The vacuum tube technique described by van Beynum and Pette (1942) was used. The tubes were incubated for 7 days at 30°C. before opening them under water.

The configuration of the lactic acid produced by the bacteria in milk was determined by the method of Orla-Jensen (1904).

## Results.

**Fermentation of cell wall substances in grass silage.**

The total amount of acid formed in grass silage was always found to be greater than the amount of sugars originally present (Table 1).

As a consequence of the high nitrogen and water contents of the grasses used, the silages often contained considerable amounts of butyric acid. However, also in the silages without butyric acid acids must have been formed — sometimes in large amounts — from cell wall substances, i.e. in a normal lactic fermentation.

**Fermentation of cell wall substances in potato pulp silage.**

Potato pulp is a by-product obtained in potato starch factories.

TABLE 1.

Demonstration of acid formation from cell wall substances.

Silage made from cut grass is denoted by C, that made with lacerated grass by L. When analysed the silages had generally been kept at 20°C. for about six months. All amounts are given in percents on dry matter.

| Silage No. | Reducing sugar obtained on hydrolysis, as glucose | Soluble carbohydrates | | | Acids formed | | | |
|---|---|---|---|---|---|---|---|---|
| | | In grass | In silage | Fermented | Butyric | Acetic | Lactic | Total |
| | % | % | % | % | % | % | % | % |
| 1 C | | 1.4 | 0.1 | 1.3 | 10.2 | 5.6 | n1) | 15.8 |
| L | | | | | 2.5 | 12.8 | n | 15.3 |
| 2 C | 26.5 | 2.8 | 0.5 | 2.3 | 8.5 | 1.3 | n | 9.8 |
| L | | | | | 0.0 | 3.6 | n | 3.6 |
| 3 C | 23.2 | 2.4 | 0.5 | 1.9 | 0.5 | 8.3 | n | 8.8 |
| L | | | | | 0.0 | 4.6 | n | 4.6 |
| 4 C | 20.2 | 3.6 | 0.4 | 3.2 | 10.9 | 6.1 | 1.8 | 18.8 |
| L | | | | | 0.2 | 10.7 | 4.1 | 15.0 |
| 5 C | 23.7 | 4.4 | | | 9.6 | 8.7 | 0.9 | 19.2 |
| L | | | | | 12.3 | 8.2 | 1.1 | 21.6 |
| 6 C | 26.0 | 13.0 | | | 4.5 | 5.5 | 7.8 | 17.7 |
| L | | | | | 0.4 | 6.0 | 16.9 | 23.3 |
| 7 C | 21.4 | 6.7 | | | 0.0 | 4.6 | 12.8 | 17.4 |
| L | | | | | 0.9 | 9.7 | 3.0 | 13.6 |
| 8 C | | 15.0 | | | 0.7 | 1.8 | 14.7 | 17.2 |
| 9 C | | 15.7 | | | 0.2 | 5.9 | 13.1 | 19.2 |
| 10 L | | 9.5 | | | 0.1 | 8.6 | 8.0 | 16.7 |

1) not determined.

It contains most of the cell walls of the potatoes and some starch in unopened cells. Although it does not contain soluble carbohydrates a lactic fermentation readily takes place in the potato pulp when it is stored in the pits. This in itself seems to prove that a lactic fermentation is indeed possible with 'insoluble' carbohydrate as a substrate.

In order to find out which carbohydrates are fermented in potato pulp we have analysed two different lots of it before and after lactic fermentation had taken place. If it is assumed that galactose is indeed mainly present as galactan, the results (Table 2) point to galactan as the principal substrate for the lactic fermentation in potato pulp. Some pectic acid is also fermented.

TABLE 2.

Change in composition of two lots of potato pulp during fermentation. The amounts of the different constituents are given in percents of dry matter.

| Constituent | Pulp A |  |  | Pulp B |  |
|---|---|---|---|---|---|
|  | Initially | After 7 months | After 12 months | Initially | After 12 months |
| Cellulose | 31.1 | 30.8 | 30.7 | 26.0 | 26.9 |
| Starch | 26.5 | 26.9 | 25.0 | 27.6 | 26.6 |
| Pectic acid | 14.0 | 11.7 | 10.2 | 14.2 | 11.0 |
| Galactan | 9.1 | 4.8 | 3.9 | 13.9 | 4.0 |
| Araban | 4.2 | 3.1 | 4.1 | 3.5 | 3.4 |
| Xylan | 1.2 | 1.2 | 1.7 | 1.5 | 1.2 |
| Lactic acid | 0.3 | 7.5 | 7.4 | 2.8 | 10.1 |
| Acetic acid | 0.1 | 3.5 | 5.0 | 0.5 | 3.0 |
| Butyric acid | 0.1 | 0.2 | 0.4 | 0.0 | 1.1 |
| Protein | 6.8 | 7.1 | 7.2 | 5.5 | 5.8 |
| Ash | 1.9 | 2.2 | 2.1 | 2.4 | 2.6 |
| Total | 95.3 | 99.0 | 97.7 | 97.9 | 95.7 |

### Isolation and identification of lactic acid bacteria fermenting galactan and galacturonic acid.

Twenty seven strains of lactic acid bacteria were isolated from several samples of potato pulp. Using methods mainly described elsewhere (DE MAN, 1956) they were all found to belong to the genus *Streptobacterium* or the genus *Betabacterium*. Five of the strains had been isolated using media containing galacturonic acid as substrate, these strains were all heterofermentative. A sixth heterofermentative strain had been isolated from media containing glucose; for the rest only homofermentative strains were isolated with these media. No doubt the homofermentative bacteria were predominant in the samples investigated.

Of the homofermentative strains seven were further identified. They were all found to be strains of *Streptobacterium casei*, as none of them could ferment melibiose or citrate, and as all formed L(+)-lactic acid. It seems likely that most of the other strains belonged to the same species.

All homofermentative strains were found to form acid in a medium, sterilised by filtration, containing a technical preparation of pectic acid (Eastman). This preparation was shown to contain, by chromatography, 10% of galactan, 3% of araban, 2% of a glucose polymer and about 0.3% of xylan. As the homofermentative strains were found unable to ferment galacturonic acid it is probable that they formed acid, not from pectic acid, but from galactan and perhaps from the other polysaccharides mentioned.

The heterofermentative strains did not form acid in the pectic acid medium. They did ferment galacturonic acid, just like the heterofermentative lactobacillus, *Betabacterium breve*, investigated by Buyze (1955). The breakdown of pectic acid in potato pulp mentioned earlier, is therefore probably caused by enzymes from yeasts or fungi present in potato pulp (Elema, 1927) or possibly by enzymes from the potato.

In a final experiment it was shown that *Streptobacterium casei* was able to ferment galactan — or at any rate galactose occurring in a polysaccharide — in potato pulp sterilised previously with ethylene oxide. After incubation the uninoculated potato pulp still proved sterile. The inoculated potato pulp was found to contain only lactobacilli. The changes reported in Table 3 and Fig. 1 must therefore have been brought about by *Streptobacterium casei*.

TABLE 3.

Determinations in potato pulp before and after fermentation by a pure culture of *Streptobacterium casei*.

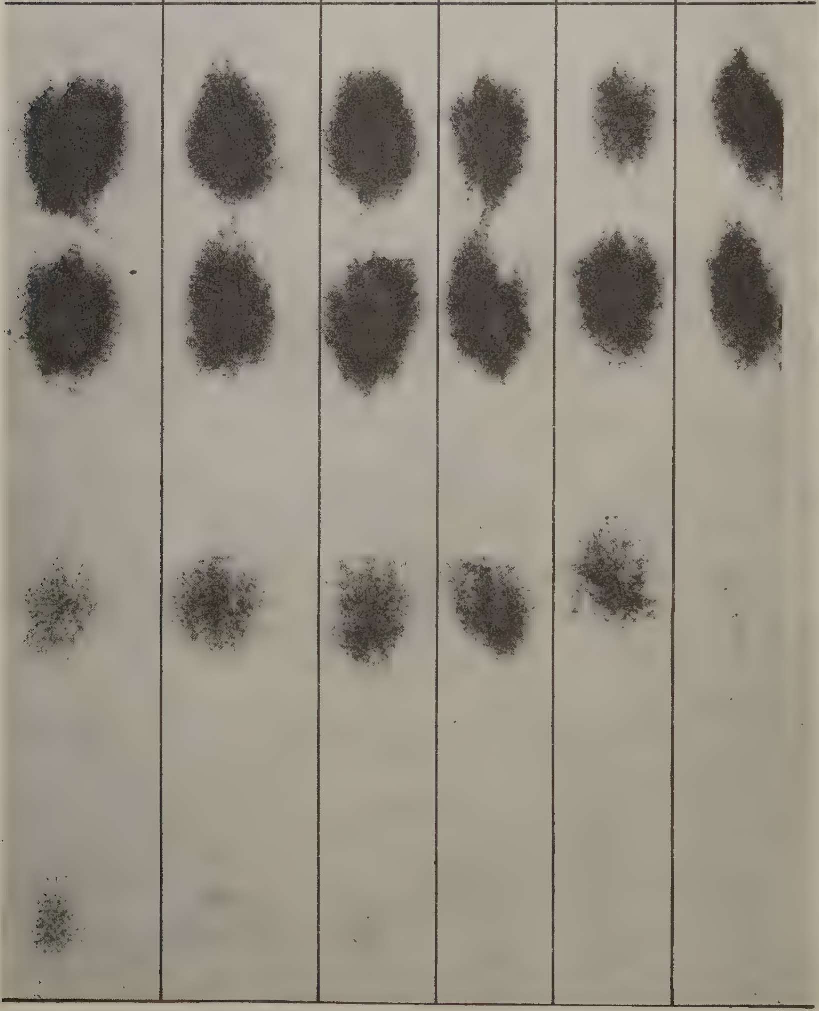
The amounts are given as percents on dry matter or as g eq. per 100 g of dry matter.

| Property determined | Untreated | Treatment: Only sterile water added | Treatment: Water added and inoculated | Treatment: Yeast extract added and inoculated | Amount of substance used or produced in the potato pulp: With water | Amount of substance used or produced in the potato pulp: With yeast extract |
|---|---|---|---|---|---|---|
| pH | 4.8 | 4.8 | 3.5 | 3.4 | | |
| volatile acid, as acetic acid, % | 0.5 | 0.6 | | 1.1 | | 0.5 |
| lactic acid, % | 0.0 | 0.1 | | 8.4 | | 8.4 |
| total of acetic and lactic acid, g eq. | | | | | | 0.098 |
| total acid titrated, g eq. | | | 0.058 | 0.104 | 0.058 | 0.104 |
| galactan, % | 22 | 22 | 18 | 13 | 4 | 9 |
| starch, % | 22 | 22 | 23 | 20 | | |
| araban, % | 3.5 | 3.3 | 3.2 | 3.5 | | |
| xylan, % | 1.0 | 1.0 | 0.9 | 1.0 | | |

FIGURE 1

Chromatogram of potato pulp hydrolysates before and after fermentation by *Streptobacterium casei*

| Reference solution | 5 $\mu$l of neutralised hydrolysate, corresponding with 200 $\mu$g of | | | | Reference solution |
|---|---|---|---|---|---|
| | unfermented potato pulp | | fermented potato pulp | | |
| galactose 50 $\mu$g<br>glucose 50 $\mu$g<br>arabinose 5 $\mu$g<br>xylose 5 $\mu$g | originally | after incubation with sterile water only | with water | with yeast extract | galactose 30 $\mu$g<br>glucose 40 $\mu$g<br>arabinose 2.5 $\mu$g<br>xylose 2.5 $\mu$g |



This bacterium is probably the most important bacterium for the acidification of potato pulp. Possibly it performs the same function in other types of silage.

## Summary.

The amount of acid formed in grass silage was greater than could have been formed from the soluble sugars present, even when only a lactic fermentation took place. This seemed to point to fermentation of cell wall substances by lactic acid bacteria.

Lactic acid fermentation in potato pulp always takes place with cell wall substances as substrates, as sugars are absent. It was found that galactose, probably occurring as galactan, and also some pectic acid were fermented in potato pulp.

Some lactobacilli were isolated from potato pulp; streptobacteria which could ferment galactan but no pectic or galacturonic acid, and betabacteria which could ferment galacturonic acid but no galactan or pectic acid.

A number of homofermentative lactobacilli were all found to belong to the species *Streptobacterium casei*. It was shown that a strain of this species could ferment galactan in potato pulp sterilised previously with ethylene oxide.

## Acknowledgement.

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(From the Laboratory for the Purification of Sera and Vaccines, National Institute of Public Health, Utrecht).

# POTENCY AND PURIFICATION OF TETANUS TOXOID OBTAINED BY MEANS OF THE MUELLER MEDIUM

by

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In several countries a casein-hydrolysate medium as first developed by MUELLER and MILLER (1947, 1954) has in the last few years been adopted for the preparation of tetanus toxoid. This medium gives considerably higher toxin yields than previous media. This method of preparing tetanus toxin is also used by the Laboratory for the Preparation of Sera and Vaccines (Dr J. L. SIRKS) of the National Institute of Public Health now.

In the past the so-called Tarozzi-medium was used for this purpose. The toxoids thus obtained were purified by ultrafiltration and fractional precipitation with ammonium sulphate (TASMAN and VAN RAMSHORST, 1952) in the manner previously developed for diphtheria toxoids (VAN RAMSHORST, 1951). The question now arose how the 'Mueller medium' toxoids could be purified most efficiently. The answer that suggested itself was: by an adaptation of the well-tried technique of ultrafiltration and precipitation.

However, the antigenic potency of the fractions obtained is even more important than the purity (expressed in Lf/mg N). Purity and potency need not necessarily run parallel, and the power to induce the production of antitoxin may also vary in different fractions of a toxoid. With respect to diphtheria toxoid this last phenomenon was described by MURATA *et al.* (1952) and had also been observed by us (but not published). The immunizing effect of fractions precipitated at low A.S. concentrations was found to be less than that of fractions precipitated at higher concentrations. In practical purification, therefore, the former have to be removed.

Consequently this was another point that had to be investigated for an efficient purification of the 'Mueller toxoid'.

The object of the experiments described below was to ascertain:

1. The salting-out curve (percentage of precipitated toxoid plotted against concentration of ammonium sulphate).
2. The purity (Lf/mg N) of the fractions obtained by fractional precipitation with A.S.
3. The immunizing potency of the fractions. This was tested after adsorption of the antigen in question on $AlPO_4$.

The data thus obtained would make it possible to decide how the 'Mueller toxoid' should be purified in practice in order to get a product that was both as pure and as potent as possible.

Finally, it was very important to know whether different batches of these toxoids vary in potency, or, in other words, whether they are all equally suitable for the preparation of a prophylactic with maximum potency. At the same time it would be possible to find out if there was any correlation between the initial titer (Lf/ml) of the crude toxoid and the antigenic potency of the purified prophylactic prepared from it.

## EXPERIMENTS.

All the batches of 'Mueller toxoid' were first concentrated by ultrafiltration through Parlodion membranes (8% solution in glacial acetic acid). This technique has already been described (VAN RAMSHORST, 1951). At the same time most of the impurities of small molecular size (amino acids etc. from the medium) were removed by this process. The concentration of the crude toxoids ranged from 11—41 Lf/ml, that of the ultrafiltration residues from 300—1.000 Lf/ml.

The concentrations thus obtained were then fractionated by precipitation with ammonium sulphate. This was done as follows: a certain quantity of ammonium sulphate was added to the fluid and the resulting precipitate removed by centrifuging, a new quantity of A.S. was then added to the fluid and the new precipitate removed in the same way, this being repeated several times. The precipitates were dissolved in water and the A.S. was removed by dialysis.

From some batches of toxoid as many as ten fractions were obtained in this manner, by raising the A.S. concentration each time by 2% (of the initial volume). The first addition was 12%, however, as this concentration yielded the first precipitate.

As it soon became clear from these experiments that the middle fractions were the purest, the fractions obtained both from low and from high A.S. concentrations being less pure, the above-mentioned batches and also a number of other batches were divided into three fractions according to the salting-out curves, *viz.*, a preliminary fraction, a middle fraction (which proved to contain most of the toxoid) and a final fraction. Generally, the A.S. concentrations used ranged from 0—15%, 15—25%, and 25—35%, respectively.

The toxoid content (Lf/ml) and the purity of the dialysed fractions obtained in these experiments having been determined, they were in many cases adsorbed on a suspension of aluminium phosphate previously prepared from alum and trisodium phosphate. The adsorbed vaccines always contained 10 Lf/ml, 3 mg $AlPO_4$/ml, merthiolate 1:10.000, pH having been adapted to 6.5. After their preparation the vaccines were immediately placed at 37°C. for 3 days to promote the adsorption and the sterilizing effect of the merthiolate.

In order to investigate the immunizing potency of these adsorbed vaccines, they were injected into groups of guinea-pigs (usually 12 to a group). The animals were given 0.5 ml subcutaneously and bled after an interval of 6 weeks. The antitoxin titers in the blood sera of all animals were then determined individually by mouse-titration. These titrations were carried out with logarithmical intervals of 100% at the Lr/100 level.

## RESULTS.

### 1. Salting-out curve.

Four separate toxoids (Nos. 26, 28, 29, 32) and a mixture (M 32) of three toxoids were divided into 10 fractions by the method described above, after which the percentage of the original flocculation value was determined for each fraction. The figures obtained are given in table 1. The picture being almost the same for each batch, fig. 1 is only given for toxoid No. 32.

Generally, about 60—70% of the toxoid is found to precipitate between 15—25% A.S. At over 30% A.S. there is no further precipitation of toxoid.

## TABLE 1.

Synopsis of purity and toxoid percentages in 10 fractions, calculated from Lf/ml, N-content, and volumen of ultrafiltered crude toxoid and fractions.

| Toxoid | | Ultrafiltered toxoid | Fractions at % A.S. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0—12 | 12—14 | 14—16 | 16—18 | 18—20 | 20—22 | 22—24 | 24—26 | 26—28 | 28—30 |
| Mixture | Lf/mgN | 500 | 660 | 860 | 1980 | 1770 | 1790 | 1630 | 1380 | 610 | 320 | 160 |
| M 32 | % Lf | — | 5.0 | 7.3 | 26.0 | 20.2 | 13.5 | 5.8 | 6.2 | 1.9 | 0.8 | 0.5 |
| 26 | Lf/mgN | 630 | 750 | 870 | 1440 | 1550 | 1650 | 1420 | 880 | 710 | 350 | 160 |
| | % Lf | — | 4.8 | 9.2 | 15.5 | 21.1 | 14.7 | 7.8 | 3.8 | 1.5 | 1.8 | 0.6 |
| 28 | Lf/mgN | 450 | 400 | 670 | 1590 | 2180 | 2000 | 1560 | 1010 | 950 | 720 | — |
| | % Lf | — | 2.6 | 1.1 | 7.9 | 23.4 | 5.5 | 19.4 | 2.5 | 5.6 | 0.8 | — |
| 29 | Lf/mgN | 410 | 430 | — | 1130 | 1720 | 1660 | 1880 | 1600 | 1480 | 970 | 220 |
| | % Lf | — | 1.6 | <0.8 | 3.6 | 14.5 | 5.7 | 24.7 | 4.3 | 9.2 | 1.0 | 0.9 |
| 32 | Lf/mgN | 440 | 500 | 920 | 1550 | 1580 | 1570 | 1220 | 960 | 590 | 280 | 80 |
| | % Lf | — | 3.5 | 7.4 | 22.7 | 23.3 | 17.4 | 5.3 | 2.4 | 3.6 | 1.2 | 0.4 |

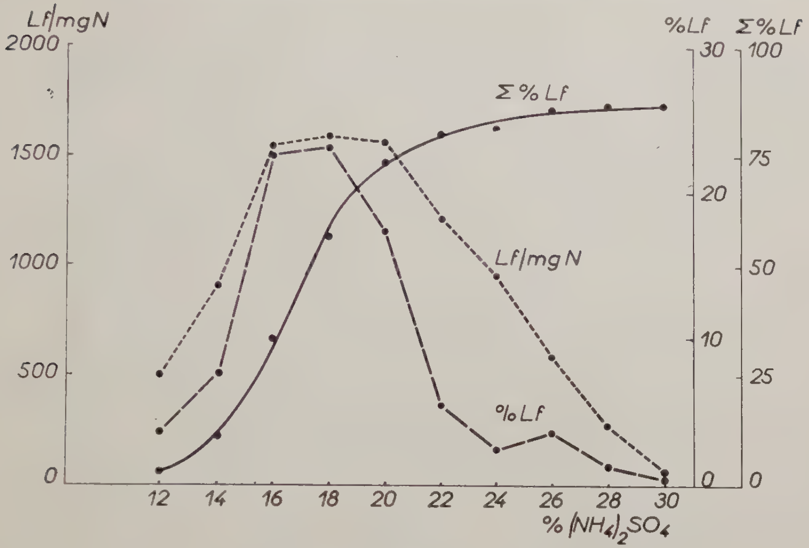

Fig. 1.

Purification of 'Mueller toxoid' No. 32.

The curves represent the percentage of original toxoid precipitated in each fraction (% Lf curve), the purity in each of these fractions (Lf/mg N curve) and the summation curve of the % Lf values (Σ% Lf).

## 2. Purity of the 10 fractions.

Table 1 also gives the purity of the fractions in Lf/mg N. For toxoid No. 32 this is also shown in fig. 1. The lower two fractions (0—12% and 12—14% A.S.) are but little purer than the crude toxoid; the next four fractions have the greatest purity; at still higher concentrations of A.S. the purity decreases rapidly, the values found becoming considerably smaller than that of the ultrafiltered crude toxoid. Therefore the limits within which the purest toxoid is precipitated are at 14—16% A.S. and 20—25% A.S., with small deviations for the different toxoids.

## 3. Separations into 3 fractions.

In view of the above data, the toxoids mentioned under 1, and 9 other toxoids, were separated into 3 fractions. Table 2 shows the percentages of toxoid and the purity of each of these fractions. In this table the figure given for the first fraction is that of the percentage of A.S. added, those for the second and third fractions represent the total concentration of A.S. in the fluid after the first and second precipitate respectively had been removed by centrifug-

TABLE 2.

Purity and toxoid percentages in 3 fractions of 14 batches of toxoid, calculated from Lf/ml, N-content, and volume of ultrafiltrated crude toxoid and fractions. For further explanations see text.

| Crude non-concentrated toxoid | | first fraction | | | second fraction | | | third fraction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Nr. | Lf/ml | % A.S. | % Lf | Lf/mg N | % A.S. | % Lf | Lf/mg N | % A.S. | % Lf | Lf/mgN |
| M 32 | 17,33,36[1] | 15 | 28 | 1090 | 25 | 57 | 1400 | 35 | 3 | 260 |
| 26 | 29 | 15 | 19 | 890 | 25 | 62 | 1320 | 35 | 3 | 240 |
| 29 | 13½ | 17 | 17 | 1000 | 26 | 51 | 1440 | 35 | 3 | 220 |
| 31 | 23 | 15 | 13 | 1160 | 24 | 63 | 1890 | 35 | 3.5 | 270 |
| 32 | 41 | 15 | 16 | 900 | 24 | 65 | 1180 | 35 | 2 | 140 |
| 33 | 12 | 15 | 7 | 500 | 24 | 70 | 1280 | 35 | 8 | 160 |
| 34 | 33 | 15 | 16 | 960 | 25 | 63 | 1620 | 35 | 2 | 140 |
| 35 | 19 | 15 | 18 | 870 | 25 | 71 | 1500 | 35 | 4 | 110 |
| 37 | 30 | 15 | 19 | 310 | 24 | 74 | 1610 | 35 | 4 | 190 |
| 40 | 12 | 15 | 10 | 600 | 24 | 67 | 1020 | 35 | 5 | 115 |
| 41 | 23 | 15 | 11 | 750 | 24 | 71 | 1400 | 35 | 2.5 | 150 |
| 43 | 19 | 15 | 10 | 630 | 24 | 62 | 1100 | 35 | 4 | 140 |
| 45 | 11 | 15 | 14 | 580 | 24 | 67 | 1270 | 35 | 5.5 | 140 |
| 49 | 28 | 15 | 19 | 840 | 24 | 65 | 1300 | 35 | 3.5 | 230 |
| Average | | | 15 | 790 | | 65 | 1380 | | 3 | 180 |

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[1] Mixture of 3 batches.

ing. The percentages of A.S. were not identical for all batches, the concentration limits for each batch being based on a previously determined salting-out curve. As the table shows, the second fraction has an average toxoid content of 65% and a fairly high degree of purity (averaging 1380 Lf/mg N). The first fraction has an average of 15% and 790 Lf/mg N, the third fraction having an average of only 3% and 180 Lf/mg N.

### 4. Antigenic potency of the fractions.

From most of the fractions mentioned under 3 an adsorbed vaccine was prepared by adsorbing the purified toxoids on $AlPO_4$ (10 Lf/ml, 3 mg $AlPO_4$/ml, merthiolate 1 : 10.000). This was injected into laboratory animals (one injection of 0.5 ml subcutaneously) and after six weeks the antitoxin content of their blood sera was determined. Table 3 shows the average titers found.

The serum titers of the individual animals, which were used in the statistical analysis (see appendix), are shown in table 4. The statistical interpretation showed that:

*a*) No correlation could be established between the toxoid content (Lf/ml) of the crude filtrate (of all batches 26—49) and the immunizing potency of the second fraction.

*b*) As regards the variance of the titers, neither the batches nor the fractions prepared from them differ significantly. The average variance of the values found for the 'inferior' third fraction is not significantly greater than in the case of the other fractions.

*c*) With respect to the first and second fractions, none of the batches 26—37 differs significantly from any of the others in potency. The potency of fraction 3 varies greatly for these batches.

*d*) Fraction 3 of most batches is significantly less potent than fractions 1 and 2, which do not differ significantly.

## Conclusions.

From the determination of the salting-out curves and purity curves of fractionated batches of 'Mueller toxoid', and of the immunizing potency of the fractions, it appeared that:

1. Fraction 1 had an average purity of 790 Lf/mg N, fraction 2 of 1380 Lf/mg N, fraction 3 of 180 Lf/mg N; fraction 2 containing on an average 65% of the toxoid.

## TABLE 3.

Antitoxin in the blood of guinea-pigs 6 weeks after 1 injection (0.5 ml subcutaneously) of the $AlPO_4$ adsorbed vaccines prepared from toxoid fractions.

| Crude toxoid | | Titers obtained with adsorbed vaccines after 1 injection | | | | | |
|---|---|---|---|---|---|---|---|
| Nr. | Lf/ml | first fraction | | second fraction | | third fraction | |
| 26 | 29 | 3.3[1] | (2.69) | 3.0 | (2.32) | 2.1 | (1.52) |
| 31 | 23 | 4.3 | (2.19) | 4.0 | (3.21) | 2.1 | (1.44) |
| 32 | 41 | 3.6 | (3.33) | 2.9 | (2.28) | 0.85 | (0.60) |
| 33 | 12 | 4.5 | (2.72) | 3.4 | (3.00) | 1.6 | (1.28) |
| 34 | 33 | 2.7 | (2.29) | 2.2 | (1.99) | 1.3 | (1.09) |
| 35 | 19 | 3.6 | (2.88) | 4.7 | (3.55) | 1.1 | (0.65) |
| 37 | 30 | 3.8 | (2.96) | 2.9 | (1.96) | 4.0 | (3.03) |
| 29 | 13½ | — | — | 5.0 | (3.68) | — | — |
| 40 | 12 | — | — | 2.4 | (1.82) | — | — |
| 41 | 23 | — | — | 4.5 | (3.04) | — | — |
| 43 | 19 | — | — | 4.2 | (3.70) | — | — |
| 45 | 11 | — | — | 3.5 | (2.57) | — | — |
| 49 | 28 | — | — | 2.7 | (2.02) | — | — |

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[1] These figures represent the arithmetical means of the titers per animal-group. The figures between brackets are the corresponding geometric means.

2. There was no significant difference in immunizing potency in guinea-pigs between the fractions 1 and 2 of any of the batches of toxoid. This leads to the important conclusion that 'Mueller toxoid' is very constant with respect to antigenic potency. Neither does the number of Lf/ml of the crude toxin — and consequently of the toxoid prepared from it — affect the potency of the purified and adsorbed final product.
3. Fraction 3 was not only very impure, but in most cases its immunizing potency per Lf was significantly inferior to that of fractions 1 and 2. Besides, the potency of the third fraction of the different batches was found to vary considerably.
4. There is a striking difference with diphtheria toxoid, where it is the fraction precipitated at a low A.S. concentration which is inferior in antigenic potency and purity.
5. For the practical preparation of tetanus vaccines one must use the second fraction, this having the greatest purity and being equally potent as the first and more potent than the third fraction.

## Summary.

A number of batches of tetanus toxin prepared by means of the 'Mueller medium' were fractionated with ammonium sulphate. The purity and the immunizing potency of the resulting fractions were determined. The fractions precipitated at low A.S. concentrations are very potent but not very pure, those precipitated at high A.S. concentrations have a low potency and are very impure. Medium concentrations (about 15—25% A.S.) yield a product which is highly satisfactory with respect to both purity and potency and forms a suitable basis for the preparation of a vaccine. The various batches of toxoid did not differ significantly in potency.

## Appendix.

The antitoxin titers obtained after immunizing guinea-pigs with adsorbed vaccines prepared from the different fractions were analysed and interpreted statistically, the analysis being based on the logarithms of the individual animal titers.

*a*) It was impossible to demonstrate any correlation between Lf/ml of the crude toxoid and the log titers of the middle fraction.

## TABLE 4.

Individual animal titers used for tables 2 and 3.

| toxoid | 26 | | | 31 | | | 32 | | | 33 | | | 34 | | | 35 | | | 37 | | | 29 | 40 | 41 | 43 | 45 | 49 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fraction | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 |
| animal titers | 2.3 | 1.6 | 1.1 | 1.6 | 1.6 | 1.1 | 6.4 | 6.4 | 0.6 | 1.1 | 1.1 | 0.6 | 0.6 | 1.6 | 2.3 | 2.3 | 9.0 | 0.1 | 2.3 | 4.5 | 3.2 | 1.6 | 2.3 | 1.1 | 1.6 | 12.8 | 0.8 |
| | 3.2 | 1.1 | 0.3 | 2.3 | 4.5 | 2.3 | 3.2 | 2.3 | 0.4 | 18.1 | 1.1 | 1.1 | 2.3 | 4.5 | 0.3 | 4.5 | 0.6 | 0.3 | 2.3 | 2.3 | 4.5 | 9.0 | 2.3 | 9.0 | 9.0 | 2.3 | 9.0 |
| | 2.3 | 2.3 | 1.1 | 9.0 | 1.1 | 2.3 | 2.3 | 0.3 | 0.1 | 4.5 | 4.5 | 1.1 | 1.1 | 1.6 | 1.1 | 3.2 | 6.4 | 0.15 | 1.1 | 0.3 | 4.5 | 4.5 | 1.1 | 2.3 | 3.2 | 1.1 | 0.4 |
| | 1.1 | 1.6 | 3.2 | 1.6 | 2.3 | 2.3 | 2.3 | 1.6 | 0.6 | 2.3 | 4.5 | 4.5 | 2.3 | 2.3 | 3.2 | 9.0 | 6.4 | 1.6 | 4.5 | 1.1 | 1.6 | 3.2 | 1.6 | 1.1 | 1.1 | 0.6 | 2.3 |
| | 2.3 | 2.3 | 0.6 | 1.1 | 9.0 | 9.0 | 2.3 | 2.3 | 1.1 | 1.1 | 4.5 | 0.6 | 2.3 | 1.1 | 0.6 | 4.5 | 9.0 | 0.8 | 0.8 | 6.4 | 0.3 | 12.8 | 2.3 | 2.3 | 0.6 | 6.4 | 1.1 |
| | 1.1 | 1.6 | 2.3 | 9.0 | 2.3 | 0.6 | 4.3 | 2.3 | 0.2 | 2.3 | 3.2 | 3.2 | 2.3 | 2.3 | 1.1 | 0.8 | 3.2 | 3.2 | 2.3 | 0.8 | 6.4 | 9.0 | 4.5 | 18.1 | 3.2 | 3.2 | 3.2 |
| | 2.3 | 1.1 | 2.3 | 9.0 | 2.3 | 1.6 | 6.4 | 3.2 | 0.3 | 1.6 | 4.5 | 1.6 | 6.4 | 1.6 | 1.1 | 3.2 | 3.2 | 0.2 | 3.2 | 4.5 | 0.8 | 1.6 | 3.2 | 1.6 | 0.8 | 1.1 | 2.3 |
| | 4.5 | 1.1 | 0.8 | 1.6 | 2.3 | 1.1 | 2.3 | 4.5 | 1.6 | 1.1 | 1.6 | 1.6 | 2.3 | 2.3 | 1.1 | 0.8 | 4.5 | 0.6 | 9.0 | 0.8 | 6.4 | 9.0 | 0.8 | 1.6 | 9.0 | 4.0 | 1.1 |
| | 3.2 | 4.5 | 1.6 | 3.2 | 6.4 | 0.4 | 4.5 | 2.3 | 2.3 | 2.3 | 4.5 | 1.6 | 4.5 | 1.6 | 1.1 | 4.5 | 1.1 | 1.6 | 6.4 | 4.3 | 4.5 | 1.1 | 0.4 | 2.3 | 1.1 | 2.3 | 1.1 |
| | 6.4 | 9.0 | 6.4 | 4.5 | 9.0 | 0.3 | 2.3 | 1.1 | 1.1 | 2.3 | 6.4 | 1.1 | 3.2 | 1.1 | 0.8 | 3.2 | 3.2 | 3.2 | 2.3 | 1.6 | 4.5 | 4.5 | 0.6 | 2.3 | 0.3 | 3.2 | 4.5 |
| | 3.2 | 6.4 | 4.5 | | 2.3 | 2.3 | | 2.3 | 1.1 | 12.8 | 3.2 | 1.1 | 2.3 | 4.5 | 1.1 | | | 0.8 | 2.3 | 1.1 | 6.4 | 6.4 | 1.1 | 4.5 | 6.4 | 1.6 | 3.2 |
| | 4.5 | 3.2 | 1.1 | | 4.5 | 2.3 | | 6.4 | | | 2.3 | 0.6 | | | 1.6 | | | | 9.0 | 6.4 | 4.5 | 4.5 | 6.4 | 9.0 | 3.2 | 4.5 | 2.3 |
| | | | | | | | | | | | | | | | | | | | | | | 1.6 | 4.5 | 4.5 | 18.1 | 2.3 | 4.5 |
| | | | | | | | | | | | | | | | | | | | | | | 1.1 | 2.3 | 2.3 | 1.1 | | |

KENDALL's rank correlation coefficient is 0.25 and does not differ significantly from nought (all batches 26—49).

*b*) The standard deviations of the log titers batches 26—37 show no significant mutual differences in any of the fractions. According to the BARTLETT-test P = 0.28 for fraction 1, P = 0.42 for fraction 2, and P = 0.32 for fraction 3. The differences between the standard deviations of batches 26—37 and of the fractions obtained from them are also non-significant (P = 0.27); in other words, the variance in the fraction does not differ significantly. The average variance being 0.127, the standard deviation of the log titers is 0.357.

*c*) As regards fractions 1 and 2, the differences between batches 26—37 are not significant; P = 0.92 and 0.44 respectively. For fraction 3 these differences are highly significant; P = 0.001. According to TUKEY's yardstick method batch 37 differs significantly (5%-point) from batches 32 and 35. There are no significant differences between the other possible combinations.

*d*) Fraction 3 of most batches is significantly less potent than fractions 1 and 2. Table 5 shows for each of these batches whether the differences between two fractions are significant at the 10% point, the 5% or the 1% point, according to SCHEFFÉ's yardstick method.

TABLE 5.

| Batch | | Difference between fractions | | |
|---|---|---|---|---|
| Lf | Nr. | 1 and 2 | 1 and 3 | 2 and 3 |
| 29 | 26 | N.S. | N.S. | N.S. |
| 41 | 32 | N.S. | 1% | 1% |
| 23 | 31 | N.S. | 10% | 10% |
| 12 | 33 | N.S. | 10% | 5% |
| 33 | 34 | N.S. | 5% | 10% |
| 19 | 35 | N.S. | 1% | 1% |
| 30 | 37 | N.S. | N.S. | N.S. |

Explanation see test.
N.S. = non-significant, 1% = significant at 1% level, etc.

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Special thanks are due to the statistician, Dr E. F. DRION, and also to Dr H. H. COHEN for his advice on the practical interpretation of the results.

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# A NOTE ON THE VEGETATIVE REPRODUCTION IN THE GENUS *LIPOMYCES*

by

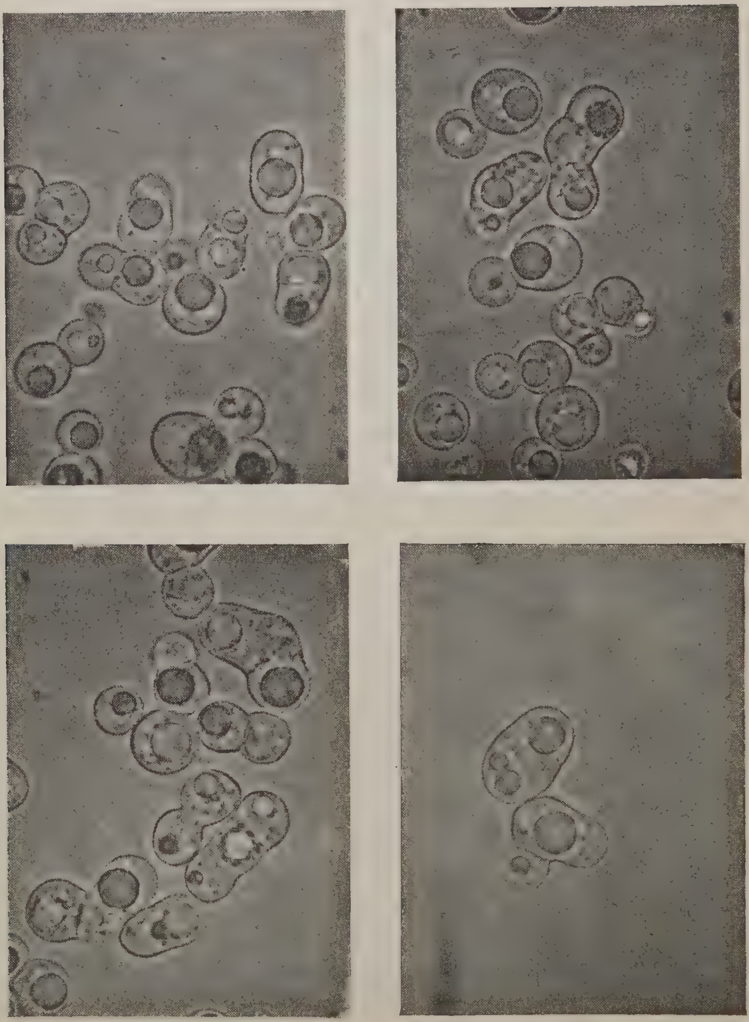
**J. P. VAN DER WALT**

(Received November 19, 1956).

Vegetative reproduction in the group of ascosporogenous soil yeasts, discovered by STARKEY (1946) and which LODDER and KREGER-VAN RIJ (1952) brought to the genus *Lipomyces* has always been reported to take place by multilateral budding only.

Recently, however, a *Lipomyces* strain was isolated locally from soil which showed reproduction by fission simultaneously with budding. This phenomenon was noted only on solid media and was best observed on Wickerham's glucose-malt-yeast extract agar at 25°C. When subculturing the strain on this medium, initial reproduction proceeds by budding while the cells remain round to short oval. This is followed by a phase when the organism reproduces by both budding and fission. After about 96 hours more or less short cylindrical cells which strongly resemble the oidia of *Schizosaccharomyces* species are first observed. These divide either directly by fission or form elongated cell forms which subsequently separate by septum formation into 3 to 5 arthrospores (Figs. 1, 2, 3 and 4). The formation of mycelium or pseudomycelium was not observed. Mrs. KREGER-VAN RIJ of the Yeast Division of the Centraal Bureau voor Schimmelcultures in Delft, in a personal communication has confirmed this phenomenon for some *Lipomyces* strains held in that collection and has also reported formation of a rudimentary pseudomycelium for one of these strains.

Beyond this feature the strain showed no other unique morphological characteristics. Subcultures readily sporulated forming typical sac-like asci with characteristic amber, oval spores. Biochemically no significant differences could be detected when



Figs. 1, 2, 3 and 4.

*Lipomyces starkeyi.* A 96 hour old culture on Wickerham's glucose-malt-yeast extract agar (800 ×).

this strain was compared with other *Lipomyces* strains. Like most other locally isolated strains, it slowly assimilated lactose. This was confirmed by Mrs. Kreger-van Rij, who, after re-examining all *Lipomyces* strains held by the Yeast Division of the Centraal Bureau, reported that it proved possible to demonstrate lactose assimilation in strains which had previously been considered as not assimilating this sugar. This is in accordance with the views of CONNELL, SKINNER and HURD (1954) that it is not possible to attach any taxonomic value to this property. Since the species *Lipomyces lipofer* (den Dooren de Jong) Lodder et Kreger-van Rij and *Lipomyces starkeyi* Lodder et Kreger-van Rij were separated on the basis of their ability or inability to assimilate lactose the former species' name, must as the American authors showed, be regarded as synonym for the latter.

SEELIGER (1956) in his studies on the urease activity of yeasts claimed that the splitting of urea was absent in the single strain of *Lipomyces starkeyi* examined by him and pointed out that the absence of this characteristic could perhaps be of diagnostic value for the genus. However, when our strain was examined, together with several other strains, a weak yet undeniable urease activity was noted on Christensen's media as prescribed by SEELIGER (1956). It would therefore seem that the inability to split urea does not serve any diagnostic purpose in characterising the genus *Lipomyces*.

However, as there are no valid reasons not to identify our strain with *Lipomyces starkeyi*, it becomes necessary to cover the additional morphological characteristics observed in it, by extension of the genus diagnosis as given by LODDER and KREGER-VAN RIJ (1952) as follows:

"Besides round to oval cells with multilateral budding, also oval to cylindrical cells, often adhering in little groups, reproducing by fission, may be formed. Cells surrounded by a slimy capsule. In older cultures, cells usually include a large fat globule. Under appropriate conditions a starch-like compound may be formed. Pseudomycelium absent or rudimentary.

Asci are formed by a vegetative cell as a sac-like protuberance of the latter, spores are oval, amber, up to 16 or perhaps more per ascus. On various media cultures have a slimy appearance.

Strictly oxidative dissimilation."

Although the discovery of septum formation establishes the mould-like characteristics of the genus *Lipomyces*, it is difficult to assess its relation to any mould forms of the Endomycetales which have been regarded as yeast progenitors. This is chiefly due to the inadequate knowledge of the life cycle of these yeasts. In no case has conjugation ever been reported for *Lipomyces starkeyi*. Neither are there any reports of germination of its ascospores being observed. However, the recent discovery (VAN DER WALT, 1956 a and b) of the yeasts *Kluyveromyces polysporus* with multispored asci and *Kluyveromyces africanus* which forms asci with as many as 16 ascospores, no longer makes the ascospore number of *Lipomyces starkeyi* unique in the yeast domain and supports the arguments of STARKEY (1946) and LODDER and KREGER-VAN RIJ (1952) to regard *Lipomyces starkeyi* as ascosporogenous.

## Acknowledgement.

The author wishes to thank Mrs KREGER-VAN RIJ of the Centraal Bureau voor Schimmelcultures for kindly confirming our observations. This communication is made with the permission of the South African Council for Scientific and Industrial Research.

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# UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. VII. MITTEILUNG

## ÜBER DEN EINFLUSS ÜBLICHER PUFFERSYSTEME ORGANISCHER SÄUREN AUF DIE AEROBE UND ANAEROBE VERGÄRUNG VON GLUCOSE DURCH DIE WEINHEFE „FENDANT" AUS JUNGEN UND ALTEN KULTUREN[1]

von

**TORSTEN WIKÉN** und **NORBERT PFENNIG**[2]

(Eingegangen am 23. Februar 1957).

Bei Untersuchungen über die anaerobe Vergärung der Glucose in 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer vom pH 4,9 durch auxo-autotrophe und auxo-heterotrophe Kulturweinhefen, welche unter streng definierten Bedingungen in synthetischen Nährlösungen ohne bzw. mit Zusatz von Wuchsstoffen gezüchtet worden waren, konnte festgestellt werden, dass die auf eine bestimmte Menge feuchter Hefe oder Hefetrockensubstanz bezogene Gärgeschwindigkeit vom Alter der Kulturen, aus denen das ,,ruhende" Zellmaterial gewonnen wurde, sehr stark abhängig ist (39). Es wurde ferner nachgewiesen, dass im erwähnten Puffersystem wesentliche Unterschiede zwischen den aus jungen und alten Kulturen geernteten Hefezellen in bezug auf die Empfindlichkeit des Zymasesystems gegen molekularen Sauerstoff bestehen (40, 41, 42, 43, 44). Die aus jungen Kulturen stammenden ,,ruhenden" Zellen sind in 0,16 M Bernsteinsäure-Succinat-Lösung hinsichtlich der alkoholischen Gärung nicht als anaerobe, sondern als mikroaerophile bis aerobe Organismen zu betrachten, weil ihr Gärvermögen in Anwesenheit geringer $O_2$-Mengen bzw. in Luft grösser ist als in $O_2$-freiem Stickstoff. Die bei jungem Zellmaterial unter streng anaeroben Bedingungen eintretende Gärungshemmung ist von der Art der Stickstoffbegasung abhängig, indem sie mit zunehmender Dauer derselben grösser wird. Sie ist ferner reversibel, indem sie durch molekularen Sauerstoff aufgehoben wird. Das Ausmass dieser Aktivierung hängt vom $O_2$-Gehalt der Gasphase der gärenden Systeme ab. Im Bereich von 0,005 bis 10 à 20 Volumen-% Sauerstoff nimmt die Gärgeschwindigkeit mit steigender $O_2$-Konzentration zu (44). Die gleichen Effekte treten auch nach Begasung des in 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer aufgeschwemmten jungen Hefematerials mit $O_2$-freiem Argon auf (41, 43). Sie werden ferner, wie wir in einem separaten Artikel ausführen werden, durch Behandlung solcher Hefezellen mit $O_2$-freiem Wasserstoff oder Kohlendioxyd hervorgerufen.

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[1] I, II, III, IV, V und VI Mitteilung: Antonie van Leeuwenhoek **17**, 209, 1951; **18**, 31, 1952; **18**, 293, 1952; **19**, 279, 1953; **20**, 385, 1954; **21**, 337, 1955.

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In Suspensionen von „ruhenden" Zellen aus alten Hefekulturen, welche mit 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer vom pH 4,9 bereitet wurden, konnten die oben beschriebenen Wirkungen nicht oder nur in geringem Ausmass festgestellt werden (40, 41, 42, 43). Die in altem Hefematerial vorhandene Zymase zeigt unter streng anaeroben Bedingungen praktisch die gleiche Leistungsfähigkeit wie in Luft oder in Gemischen von Stickstoff und kleinen Mengen Sauerstoff.

Die oben in aller Kürze beschriebenen Gäreigenschaften der Kulturweinhefen sind an bestimmte Abschnitte der S-förmigen Wachstums- bzw. Vermehrungskurven, d.h. an bestimmte Phasen in der Entwicklung der Hefekulturen, gebunden. Sie treten somit unabhängig von den im Laufe des Wachstums bzw. der Vermehrung der Hefezellen stattfindenden Veränderungen in der Zusammensetzung des Kultursubstrats ein (42, 43). Es sei hier ferner erwähnt, dass die gleichen Erscheinungen auch bei der untergärigen Bierhefe „Müllerbräu" festgestellt werden konnten (42, 43).

Gärungen, welche in 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer mit Gemischen von Zellen aus jungen und alten Hefekulturen angesetzt wurden, wiesen sowohl unter aeroben als auch unter anaeroben Bedingungen einen intermediären Verlauf auf. Ihre Geschwindigkeiten liessen sich annähernd additiv aus den Werten berechnen, welche für Gärungen ermittelt wurden, die nur mit Zellmaterial aus jungen bzw. nur mit solchem aus alten Kulturen parallel durchgeführt wurden. Die Tatsache, dass das junge und alte

Zellmaterial in Gemischen seine individuellen Gäreigenschaften beibehält, deutet darauf hin, dass diese auf Faktoren zurückzuführen sind, welche sich unter den betreffenden experimentellen Bedingungen von den intakten Zellstrukturen nicht trennen lassen (43).

Die Tatsache, dass bei jungem Zellmaterial der Wein- und Bierhefen in 0,16 M Bernsteinsäure-Natriumsuccinat-Lösung die Vergärung der Glucose in $O_2$-freiem Stickstoff (Argon, Wasserstoff und Kohlendioxyd) gehemmt ist im Vergleich zu den parallelen Gärungen in Stickstoff-Sauerstoff-Gemischen oder Luft, ist besonders interessant im Hinblick auf den sogenannten PASTEUR-MEYERHOF-Effekt, der im Kohlehydratstoffwechsel der verschiedensten Zellen auftritt und darin besteht, dass in Anwesenheit des molekularen Sauerstoffs die Gärung (Zuckerdissimilation ohne $O_2$-Aufnahme) zu Gunsten der Atmung (Zuckerdissimilation mit $O_2$-Aufnahme) zurückgedrängt wird (9, 17). Bei den aus jungen Kulturen stammenden Hefezellen lässt sich ja im erwähnten Puffer der entgegengesetzte Effekt nachweisen, indem die Gärung im Bereich von 0,005 bis 10 à 20 Volumen-% Sauerstoff mit zunehmender Konzentration dieses Gases gefördert wird.

Die beschriebene Wirkung der Bernsteinsäure-Natriumsuccinat-Lösung auf die alkoholische Gärung bei jungem Zellmaterial verschiedener Hefen ist auch im Hinblick darauf bemerkenswert, dass von RUNNSTRÖM u. Mitarb. (33, 34) sowie BRANDT (5, 6, 7) stets die Indifferenz dieses Puffers gegenüber intakten Zellen der Bäcker- und Brauereihefen hervorgehoben wurde. Ferner wiesen SUOMALAINEN und OURA (37) kürzlich nach, dass die Zellen einer Bäckerhefe keine Bernsteinsäure und diejenigen einer Brauereihefe im Laufe von 30 Min. bis 6 Std. nur verhältnismässig geringe Mengen dieser Säure aufzunehmen vermögen, und dies unabhängig davon, ob die zur Bereitung der Zellsuspensionen verwendete Lösung die Säure allein (0,2 N) oder in Mischung mit ihrem Natriumsalz (pH = 4,7) enthält. Ueber die Qualität des untersuchten Hefematerials erwähnen SUOMALAINEN und OURA (37) nur, dass es sich in ihren Versuchen um „freshly washed and filtered brewer's or baker's yeast" handelt. Es ist aber anzunehmen, dass eine sogenannte „reife" („completed", „ripe" oder „stationary") Bäckerhefe, d.h. eine Hefe mit völlig oder fast völlig abgeschlossener Knospung, zur Verwendung kam. Bezüglich der Bierhefe ist es schwieriger, etwas über den physiologischen Zustand des untersuchten Zellmaterials auszusagen. Es ist aber wahrscheinlich, dass es sich auch in diesem Fall um eine verhältnismässig ,,reife'' Hefe handelte. Runnström u. Mitarb. (33, 34) sowie Brandt (5, 6, 7) haben zum Teil ,,reife'' Bäckerhefe, zum Teil aber auch solche mit ,,sehr intensiver'' (,,very intense'') oder ,,nicht völlig abgeschlossener'' (,,not completely terminated'') Knospung untersucht. Sowohl die unter starker Belüftung erzeugte ,,fertige Presshefe'' als auch die verschiedenen unter schwacher oder starker Belüftung gewonnenen ,,Stellhefen'' stammten aus industriellen Massenkulturen. Die Bierhefe wurde von den erwähnten Forschern aus Brauereien bezogen und befand sich somit wahrscheinlich in einem verhältnismässig ,,reifen'' Stadium.

Zusammenfassend kann wohl gesagt werden, dass die oben zitierten Untersuchungen, in welchen die völlige Indifferenz oder grosse Trägheit des Bernsteinsäure-Succinat-Systems bei der Gärung und anderen physiologischen Prozessen der Hefen hervorgehoben wurde, mit Hilfe eines Zellmaterials ausgeführt wurden, das in seinem physiologischen Zustand am ehesten demjenigen entspricht, welches von Wikén und Richard (39, 40, 41, 42, 43, 44) als ,,alt'' oder ,,sehr alt'' bezeichnet worden ist. Wie bereits dargestellt, konnte bei diesem Hefematerial kein oder höchstens ein sehr schwacher Bernsteinsäure-Succinat-Effekt festgestellt werden. Die zitierten Arbeiten von Wikén und Richard zeigen nun, dass aus den Ergebnissen, welche in Versuchen mit Hefezellen aus alten Kulturen gewonnen werden, nicht ohne weiteres Schlussfolgerungen über den Einfluss organischer Säuren und deren Puffersysteme auf die Gärung und weitere Lebensprozesse bei den in jungen Hefekulturen vorhandenen Zellen gezogen werden dürfen. Das von Wikén und Richard als ,,jung'' bezeichnete Zellmaterial, bei welchem die Wirkung des 0,16 M Bernsteinsäure-Natriumsuccinat-Puffers vom pH 4,9 deutlich zum Vorschein kommt, entspricht der Induktionsperiode oder Anpassungsphase der Hefekulturen (= ,,period of cellular adjustment'' = ,,initial stationary phase'' + ,,phase of accelerating growth and multiplication rate'').

Suomalainen und Oura (37) haben ferner das Vermögen des alten Hefematerials geprüft, Citronensäure, Weinsäure und Milchsäure sowie Essigsäure, Propionsäure und Buttersäure aufzunehmen. Es ergab sich, dass die Fettsäuren sowohl von Bäcker- wie von Brauereihefe sehr rasch aufgenommen werden, während Citronensäure und Weinsäure bei der ersten Hefe gar nicht und bei der letzten sogar mit geringerer Geschwindigkeit als die Bernsteinsäure aus den Medien verschwinden. Milchsäure wird von beiden Hefesorten aufgenommen und zwar rascher als Bernsteinsäure, Citronensäure und Weinsäure aber langsamer als die Fettsäuren.

Hägglund und Augustson (8) haben u.a. den Einfluss von Essigsäure-Natriumacetat-Lösungen verschiedener Konzentration auf die Vergärung der Glucose und Saccharose durch Presshefe bei pH-Werten zwischen durchschnittlich 3,00 und 6,40 untersucht. Sie stellten einen optimalen pH–Bereich von 4,5–5,5 fest, wobei das Optimum mit steigender Konzentration des Puffers nach der neutralen Seite verschoben wurde. Sie sind der Ansicht, dass die alkoholische Gärung gegen freie Essigsäure ausserordentlich empfindlich ist. Hinsichtlich der Qualität der verwendeten Hefe wird nur erwähnt, dass sie „immer von bester Güte" war.

In Untersuchungen über verschiedene Weinheferassen („Dézaley", „Sion", „Malvoisie") hat Porchet (31) nachgewiesen, dass die Vergärung von Traubensäften, welche 185 g Zucker pro Liter enthalten, nach Zusatz von 0,6 Volumen-% Essigsäure (98 %) stark gehemmt wird und in Gegenwart von 0,8 Volumen-% der gleichen Säure praktisch aufhört („fermentation très lente et incomplète", „la quantité limite"). Es handelte sich in diesen Experimenten nicht um „ruhendes" Zellmaterial, sondern um wachsende und sich vermehrende Zellen. Porchet (31) gibt keine pH-Werte an. Bezüglich der Hefequalität wird nur gesagt, dass als Impfmaterial „des levures centrifugées en pleine activité" zur Verwendung kamen.

Schliesslich sei bemerkt, dass in den Arbeiten von Suomalainen und Oura (37), Hägglund und Augustson (8) sowie Porchet (31) nicht angegeben wird, ob die Versuche unter aeroben oder anaeroben Bedingungen ausgeführt wurden.

Wie bereits erwähnt, haben Wikén und Richard (39, 40, 41, 42, 43, 44) beim Feststellen des Bernsteinsäure-Succinat-Effekts einen 0,16 M Puffer, hergestellt durch Neutralisieren von 200 ml 0,5 M Bernsteinsäurelösung mit 236 ml 0,5 M Natronlauge und Zufügen von 189 ml dest. Wasser, verwendet. Wie beispielsweise aus den Untersuchungen von Perquin (30) bzw. MacLeod und Snell (18, 19) sowie Barker (2) über Wachstum und Stoffwechsel von *Aspergillus niger* bzw. Milchsäurebakterien und Methanbakterien hervorgeht, ist die physiologische Aktivität der Zellen zahlreicher Mikroorganismen vom Konzentrationsverhältnis verschiedener Kationen im Medium stark abhängig, wobei das Verhältnis K/Na unter Umständen eine grosse Rolle spielt. Ferner wurde durch die Untersuchungen von Lardy und Ziegler (14) nachgewiesen, dass Kaliumionen für die Reversibilität der von Phosphoenoltransphosphorylase (Phosphokinase 2) katalysierten Dephosphorylierung der Enolbrenztraubensäurephosphorsäure in Anwesenheit der Adenosindiphosphorsäure von ausschlaggebender Bedeutung sind. Es schien uns deshalb wichtig festzustellen, ob der Bernsteinsäure-Succinat-Effekt im Grunde eher auf die Abwesenheit der Kaliumionen bzw. auf die hohe Konzentration der Natriumionen in der für die Gärversuche verwendeten Pufferlösung zurückzuführen sei.

Es schien uns ferner bedeutungsvoll zu prüfen, ob der von WIKÉN und RICHARD festgestellte Effekt für das Bernsteinsäure-Succinat-System spezifisch ist, oder auch durch Puffersysteme anderer organischer Säuren hervorgerufen werden kann. In der vorliegenden Arbeit wurde der Einfluss von DL-Aepfelsäure-Malat-, Fumarsäure-Fumarat-, Citronensäure-Citrat-, D-Weinsäure-Tartrat- und Essigsäure-Acetat-Lösungen auf die Vergärung der Glucose durch „ruhende" Zellen aus jungen „Fendant"-Kulturen unter aeroben und anaeroben Bedingungen untersucht. Abgesehen von D-Weinsäure, deren Kaliumsalz sehr schwerlöslich ist, und Essigsäure, wurden die Pufferlösungen in bestimmten Experimenten durch Neutralisieren der Säurelösungen mit Kaliumhydroxyd, in anderen durch Neutralisieren dieser Lösungen mit Natriumhydroxyd bereitet. Im Falle der D-Weinsäure und Essigsäure kam als Neutralisationsmittel nur Natronlauge zur Verwendung. Der pH-Wert betrug durchgehend ca. 4,9. Zum Vergleich dienten Gärungen, welche in Bernsteinsäure-Succinat-Puffer oder dest. Wasser durchgeführt wurden. Bekanntlich spielen die erwähnten Säuren zum Teil eine wichtige Rolle im Di- und Tricarbonsäurecyclus. Ferner treten sie in grösseren oder kleineren Mengen als Bestandteile von unvergorenen und vergorenen Trauben-, Birnen- und Aepfelsäften auf (BENVEGNIN, CAPT und PIGUET (3), AMERINE und JOSLYN (1), BÜCHI (4), RENTSCHLER und TANNER (32), TANNER und RENTSCHLER (38)).

Es schien uns weiterhin unerlässlich, die Abhängigkeit des Bernsteinsäure-Succinat-Effekts von der Konzentration des Puffers beim pH 4,9 näher zu untersuchen. Dabei kam nicht nur Zellmaterial aus jungen, sondern auch solches aus alten Hefekulturen zur Verwendung. Die Puffersysteme DL-Aepfelsäure-Malat, Fumarsäure-Fumarat, Citronensäure-Citrat, D-Weinsäure-Tartrat und Essigsäure-Acetat wurden ebenfalls in verschiedenen Konzentrationen geprüft.

## METHODIK.

Die zur Gewinnung des Hefematerials und zur Durchführung der Gärversuche verwendeten Methoden wurden bereits von WIKÉN und RICHARD (39, 41, 42, 43) ausführlich beschrieben. Hier sei erwähnt, dass die auxo-autotrophe „Fendant"-Hefe in Nährlösung C ohne irgend einen Zusatz von Wuchsstoffen gezüchtet wurde. Dieses Substrat enthält neben Mineralsalzen und Kaliumcitrat-Puffer vitaminfreies Caseinhydrolysat als Stickstoffquelle und Glucose als Kohlenstoff- und Energiequelle. Es hat nach dem Sterilisieren einen pH-Wert von 4,8–5,0. Als Kulturgefässe dienten Fernbach-Kolben aus Jena-Geräteglas 20 von ca. 1800 ml Inhalt. Diese enthielten 250 ml Nährlösung, wobei die Tiefe der Flüssigkeitsschicht etwa 10 mm betrug. Die Kulturen wurden bei 20°C. oder 23°C. bebrütet.

Die Bebrütungszeit der jungen Kulturen schwankte zwischen 15½ und 17 Std. (vgl. unten), während diejenige der alten Kulturen 69 Std. betrug.

Sämtliche Gärversuche wurden bei 25°C. in der volumetrischen Apparatur nach VON EULER, MYRBÄCK, NILSSON und ALM (siehe NILSSON (22)) ausgeführt. Dabei kamen stets Suspensionen von „ruhenden" Hefezellen zur Verwendung. Die Bereitung dieser Suspensionen wird unten in der Beschreibung der einzelnen Versuche näher ausgeführt. Als Gärgefässe dienten Erlenmeyer-Kolben aus Pyrex-Glas von etwa 50 ml Gesamtvolumen mit einer seitlich eingeschliffenen drehbaren Ampulle. Sie enthielten 2,0 ml Hefesuspension im Hauptraum und 1,0 ml Glucoselösung in der Seitenampulle. Abgesehen von dem in Abb. 4 aufgeführten Versuch entsprach dies 300 mg gewaschener frischer Hefe und 200 mg wasserfreiem Zucker. Im erwähnten Versuch betrug die Hefemenge zufälligerweise nur 242 mg pro Gärgefäss.

Die Geschwindigkeit der aeroben Kohlendioxydbildung wurde in Luft und diejenige der anaeroben Gärung in $O_2$-freiem Stickstoff ermittelt. Vor dem Einkippen der Glucoselösungen aus den Seitenampullen wurden diese sowie die in den Haupträumen der Gärgefässe vorhandenen Hefesuspensionen in sämtlichen Versuchen während 120 Min. in ruhender Luft (aerobe Gärung) bzw. in strömendem $O_2$-freiem Stickstoff (anaerobe Gärung) geschüttelt. Der zur Begasung der Gärkolben verwendete Stickstoff wurde von Sauerstoff befreit, indem wir das Gas durch alkalische Pyrogallollösung und anschliessend über rotglühendes reduziertes Kupfer im elektrischen Ofen bei 400–450°C. strömen liessen. In einigen Fällen wurde der Pyrogallollösung eine alkalische Natriumhyposulfitlösung vorgeschaltet.

Zur Herstellung der Pufferlösungen wurden folgende Präparate verwendet: Bernsteinsäure p.a. (Merck, Darmstadt), Citronensäure p.a. (Analar, British Drug Houses Ltd.), D-Weinsäure p.a. (Merck, Darmstadt), DL-Aepfelsäure (Merck, Darmstadt), Fumarsäure (Merck, Darmstadt) und Essigsäure p.a. (Eisessig, 99–100%, Merck, Darmstadt) sowie Kaliumhydroxyd puriss. p.a. (Merck, Darmstadt) und Natriumhydroxyd puriss. p.a. (Merck, Darmstadt, oder EKA, Bohus). Der pH-Wert der Pufferlösungen schwankte zwischen 4,86 und 4,93. Zur Bereitung der Glucoselösungen diente D(+)-Glucose (vitaminfrei, Hoffmann-La Roche, Basel).

Als Mass des Gärvermögens der „ruhenden" Hefezellen werden in den Abb. 1–12 sowie im Text die abgelesenen Kohlendioxydvolumina ohne Umrechnung auf Normalbedingungen angegeben. Eine Umrechnung erübrigt sich, da wir nur die Geschwindigkeiten gleichzeitig durchgeführter und somit unter identischen Temperatur- und Druckbedingungen verlaufener Gärungen miteinander vergleichen.

## VERSUCHSERGEBNISSE.

### Orientierender Versuch mit Zellmaterial aus jungen Hefekulturen.

Die Vergärung der Glucose durch Hefe aus jungen Kulturen wurde parallel in Citronensäure-Kaliumcitrat- bzw. DL-Aepfelsäure-Kaliummalat- und D-Weinsäure-Natriumtartrat-Puffer unter aeroben (Luft) und anaeroben Bedingungen ($O_2$-freier Stickstoff) gemessen. Das Zellmaterial stammte aus 16 Std. alten Kulturen und wurde 2mal in dest. Wasser und 1mal in der betreffenden Pufferlösung gewaschen. Sämtliche Pufferlösungen waren 0,16 M bei einem pH-Wert von 4,9. Die Versuchsergebnisse gehen aus Abb. 1 hervor.

Es ist ersichtlich, dass die geprüften Pufferlösungen sich grundsätzlich anders verhalten als der Bernsteinsäure-Kaliumsuccinat-Puffer der gleichen Stärke und des gleichen pH-Wertes. Erstens unterscheidet sich die in $O_2$-freiem Stickstoff gemessene Gärgeschwindigkeit verhältnismässig wenig von derjenigen, die im gleichen Puffer in Luft festgestellt werden konnte. Beispielsweise betrugen die Kohlendioxydmengen, welche unter anaeroben bzw. aeroben Bedingungen nach 150 Min. entwickelt worden waren, für

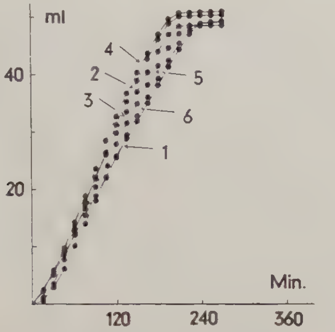


Abb. 1. Aerobe und anaerobe Vergärung der Glucose in 0,16 M Pufferlösungen bei pH 4,9 durch ruhende Zellen aus 16 Std. alten Kulturen der Weinhefe „Fendant".

| | Aerob | Anaerob |
|---|---|---|
| Citronensäure-Kaliumcitrat: | 1 | 2 |
| DL-Aepfelsäure-Kaliummalat: | 3 | 4 |
| D-Weinsäure-Natriumtartrat: | 5 | 6 |

den Citronensäure-Citrat-Puffer 39,0 bzw. 32,0 ml, für den Aepfelsäure-Malat-Puffer 40,5 bzw. 37,1 ml und für den Weinsäure-Tartrat-Puffer 32,9 bzw. 34,9 ml. Zweitens ist nur im Falle des Weinsäure-Tartrat-Puffers die nach Begasung mit $O_2$-freiem Stickstoff gemessene durchschnittliche Gärgeschwindigkeit kleiner als diejenige, die nach Behandlung der Hefesuspensionen mit Luft ermittelt wurde. In Citronensäure-Citrat- und Aepfelsäure-Malat-Puffer lässt sich dagegen unter anaeroben Bedingungen durchschnittlich eine grössere Gärgeschwindigkeit feststellen als unter aeroben.

### Vergleich der Wirkung der Puffersysteme DL-Aepfelsäure-Kaliummalat, Citronensäure-Kaliumcitrat, Fumarsäure-Kaliumfumarat und D-Weinsäure-Natriumtartrat mit derjenigen des Bernsteinsäure-Kaliumsuccinat-Puffers in Suspensionen von Zellen aus jungen Hefekulturen.

Es wurden zwei Versuche mit Hefe aus 16 bzw. 15½ Std. alten Kulturen durchgeführt, wobei im ersten Versuch Fumarsäure-Kaliumfumarat- und DL-Aepfelsäure-Kaliummalat-Lösungen, im zweiten Citronensäure-Kaliumcitrat- und D-Weinsäure-Natriumtartrat-Lösungen neben Bernsteinsäure-Kaliumsuccinat-Puffer als Suspensions- bzw. Lösungsmittel für die Hefe bzw. den Zucker dienten.

Die pH-Werte der 0,16 M Pufferlösungen betrugen in der erwähnten Reihenfolge 4,90, 4,86, 4,88, 4,90 und 4,93. Das Zellmaterial wurde 2mal in dest. Wasser und anschliessend 3mal im betreffenden Puffer gewaschen, wobei es beim zweiten Waschen mit Pufferlösung in dieser während 30 Min. aufbewahrt wurde. Die nach dem üblichen Schütteln der Hefesuspensionen in ruhender Luft bzw. strömendem $O_2$-freiem Stickstoff (120 Min.) und Einkippen der Glucoselösung gemessenen Gärgeschwindigkeiten gehen aus Abb. 2 und Abb. 3 hervor. Die in Abb. 2 aufgeführten Gärkurven bestätigen die Ergebnisse des orientierenden Versuches (Abb. 1) in bezug auf das Verhalten der im Aepfelsäure-Malat-Puffer aufgeschwemmten Hefezellen. Diese zeigen in $O_2$-freiem Stickstoff eine grössere Gärgeschwindigkeit als in Luft, wobei der Unterschied verhältnismässig klein ist (nach 150 Min. 36,4 bzw. 32,9 ml Kohlendioxyd). Der Fumarsäure-Kaliumfumarat-Puffer weist im Prinzip die gleiche Wirkung auf wie das System Aepfelsäure-Malat, wobei aber die Differenz zwischen den anaerob und aerob gemessenen Gärgeschwindigkeiten relativ gross ist (nach 150 Min. 36,1 bzw. 29,6 ml Kohlendioxyd). Gegenüber den mit Aepfelsäure-Malat- bzw. Fumarsäure-Fumarat-Lösung behandelten Hefezellen, welche in Luft die gleiche Gärfähigkeit zeigen, sind die in Bernsteinsäure-Kaliumsuccinat-

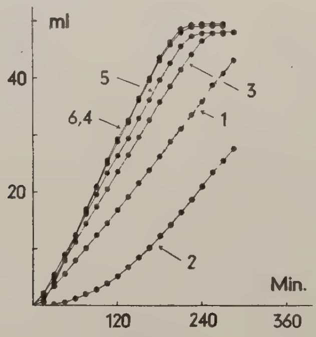


Abb. 2. Aerobe und anaerobe Vergärung der Glucose in 0,16 M Pufferlösungen bei pH 4,9 durch ruhende Zellen aus 16 Std. alten Kulturen der Weinhefe „Fendant".

| | Aerob | Anaerob |
|---|---|---|
| Bernsteinsäure-Kaliumsuccinat: | 1 | 2 |
| Fumarsäure-Kaliumfumarat: | 3 | 4 |
| DL-Aepfelsäure-Kaliummalat: | 5 | 6 |

Puffer gewaschenen und aufgeschwemmten Zellen nicht nur unter anaeroben sondern auch unter aeroben Bedingungen durch eine starke Hemmung des Gärvermögens gekennzeichnet, wobei wie immer die Gärgeschwindigkeit in $O_2$-freiem Stickstoff wesentlich kleiner ist als in Luft (nach 150 Min. 8,4 bzw. 21,6 ml Kohlendioxyd).

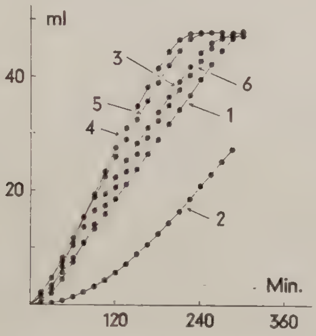


Abb. 3. Aerobe und anaerobe Vergärung der Glucose in 0,16 M Pufferlösungen bei pH 4,9 durch ruhende Zellen aus 15½ Std. alten Kulturen der Weinhefe „Fendant".

| | Aerob | Anaerob |
|---|---|---|
| Bernsteinsäure-Kaliumsuccinat: | 1 | 2 |
| Citronensäure-Kaliumcitrat: | 3 | 4 |
| D-Weinsäure-Natriumtartrat: | 5 | 6 |

Aus Abb. 3 geht hervor, dass sich die Ergebnisse des orientierenden Versuches auch in bezug auf den Einfluss der Citronensäure-Kaliumcitrat- und Weinsäure-Natriumtartrat-Lösungen auf die Vergärung der Glucose durch Zellmaterial aus jungen Kulturen der Weinhefe „Fendant" im Prinzip reproduzieren lassen. Im Citronensäure-Citrat-Puffer ist die Gärgeschwindigkeit in $O_2$-freiem Stickstoff grösser als in Luft (nach 150 Min. 35,0 bzw. 28,4 ml Kohlendioxyd), während im Weinsäure-Tartrat-System die anaerob ermittelte Gärfähigkeit kleiner ist als die aerob ermittelte (nach 150 Min. 26,5 bzw. 32,6 ml Kohlendioxyd). Diesen beiden Puffersystemen gegenüber zeigt die Bernsteinsäure-Succinat-Lösung eine hemmende Wirkung auf das Gärvermögen der Hefe, und zwar sowohl unter aeroben wie auch unter anaeroben Bedingungen (nach 150 Min. 23,8 bzw. 8,9 ml Kohlendioxyd).

Unter den von uns gewählten experimentellen Bedingungen lassen sich mithin zwei Gruppen von Pufferlösungen unterscheiden, und zwar einerseits die Fumarsäure-Fumarat- bzw. Aepfelsäure-

Malat- und Citronensäure-Citrat-Systeme, in welchen bei jungem Zellmaterial die anaerob ermittelte Gärgeschwindigkeit grösser ist als die aerob gemessene, andererseits die Bernsteinsäure-Succinat- und Weinsäure-Tartrat-Systeme, in welchen beim gleichen Hefematerial unter anaeroben Bedingungen eine kleinere Gärgeschwindigkeit festgestellt werden kann als in Luft. Im Vergleich zu den Fumarsäure-Fumarat-, Aepfelsäure-Malat-, Citronensäure-Citrat- und Weinsäure-Tartrat-Lösungen übt der Bernsteinsäure-Succinat-Puffer (0,16 M, pH = 4,9) einen hemmenden Einfluss auf die aerobe und anaerobe Vergärung der Glucose durch Hefe aus jungen Kulturen aus, wobei die unter anaeroben Bedingungen hervorgerufene Hemmung besonders stark ist.

### Die Konzentrationsabhängigkeit der Wirkung des Bernsteinsäure-Kaliumsuccinat-Puffers auf den aeroben und anaeroben Glucoseumsatz in Suspensionen von Hefezellen aus jungen Kulturen.

Es wurden drei Experimente ausgeführt, wobei Hefe aus 16½ bzw. 17 und 16½ Std. alten Kulturen zur Verwendung kam. Das Zellmaterial wurde 2mal in dest. Wasser, sodann 1mal in 0,16 M

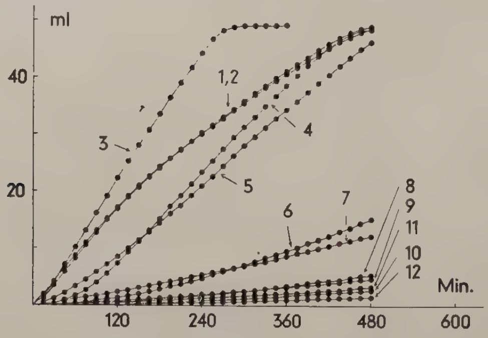


Abb. 4. Aerobe und anaerobe Vergärung der Glucose in dest. Wasser und in Bernsteinsäure-Kaliumsuccinat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Dest. Wasser: | | 1 | 2 |
| Bernsteinsäure-Kaliumsuccinat | 0,1 M: | 3 | 4 |
| „ | 0,2 M: | 5 | 6 |
| „ | 0,3 M: | 7 | 8 |
| „ | 0,4 M: | 9 | 10 |
| „ | 0,5 M: | 11 | 12 |

Bernsteinsäure-Kaliumsuccinat-Puffer und wiederum 1mal in dest. Wasser gewaschen. Es wurde in dest. Wasser aufgeschwemmt. Die so bereiteten Hefesuspensionen wurden mit Bernsteinsäure-Succinat-Lösungen der doppelten für den Gärversuch gewünschten Molarität im Volumenverhältnis 1 : 1 versetzt. Vor dem Einkippen der Zuckerlösungen wurden die Ansätze in üblicher Weise während 120 Min. in Luft bzw. in strömendem $O_2$-freiem Stickstoff geschüttelt. Der pH-Wert betrug in sämtlichen Fällen 4,9. Geprüft wurden Bernsteinsäure-Kaliumsuccinat-Lösungen der folgenden Konzentrationen: 0,0 (dest. Wasser) bzw. 0,1, 0,2, 0,3, 0,4 und 0,5 M (Abb. 4); 0,0 (dest. Wasser) bzw. 0,04, 0,08, 0,12, 0,16 und 0,20 M (Abb. 5); 0,11 bzw. 0,12, 0,13, 0,14, 0,15 und 0,16 M (Abb. 6).

Aus Abb. 4 geht hervor, dass die Vergärung der Glucose in dest. Wasser durch Zellen aus jungen ,,Fendant''-Kulturen mit der gleichen Geschwindigkeit vor sich geht unabhängig davon, ob der Prozess unter aeroben oder anaeroben Bedingungen durchgeführt wird. Ferner ist ersichtlich, dass die für diese Hefesuspensionen erhaltenen Gärkurven zwei Phasen aufweisen, von denen die erste eine durchschnittliche Gärgeschwindigkeit von 2,1 ml Kohlendioxyd pro 15 Min. und die letzte eine solche von 1,4 ml pro 15 Min. zeigt. Der Übergang der ersten Phase in die zweite wird nicht durch einen Knickpunkt, d.h. nicht durch ein scharfes Abbiegen der Gärkurve gekennzeichnet. Man darf vielmehr von einem Übergangsbereich sprechen, welcher in der Zeitspanne zwischen 150 Min. (in Luft bzw. Stickstoff 21,0 bzw. 20,7 ml Kohlendioxyd) und 210 Min. (in Luft bzw. Stickstoff 27,4 bzw. 27,3 ml Kohlendioxyd) liegt. Im erwähnten Bereich sinkt die Gärgeschwindigkeit verhältnismässig rasch von 2,0 auf 1,4 ml pro 15 Min., und dies zwar gerade um den Punkt herum (180 Min.), wo die Hälfte des zugesetzten Zuckers vergoren worden ist. Nach 180 Min. hatten sich in Luft 24,4 und in Stickstoff 24,1 ml Kohlendioxyd entwickelt, während nach beendeter Gärung 49,0 bzw. 48,6 ml festgestellt werden konnten. Dieses Verhalten des jungen Zellmaterials bei der Vergärung der Glucose in dest. Wasser erinnert an den Gärtypus, der besonders von Nilsson u. Mitarb. (23, 24, 25, 26, 27, 28) für die desorganisierten Zymasesysteme beschrieben worden ist, welche vorliegen in strukturlosen Macerations- oder Pressäften sowie in autolysierten Trockenhefepräparaten und der sogenannten intakten Trockenhefe nach deren Erhitzung oder Behandlung mit organischen Lösungsmitteln wie Aceton, Aethylalkohol, Toluol, Benzol und Tetrachlorkohlenstoff, mit Gallensäuren wie Desoxycholsäure und Taurocholsäure, oder mit Saponinen wie Digitonin. Im Vergleich zu der in dest. Wasser suspendierten Hefe weisen die in 0,1 M Bernsteinsäure-Succinat-Lösung aufgeschwemmten Zellen unter aeroben Bedingungen eine kräftige Stimulierung auf. Die Glucose wird von dieser Hefe längs einer annähernd geradlinigen Kurve vollständig vergoren, wobei die durchschnittliche Gärgeschwindigkeit 2,8 ml Kohlendioxyd pro 15 Min. (Zeitspanne 15–255 Min.) beträgt. Bei den übrigen in Luft geprüften Konzentrationen tritt dem dest. Wasser gegenüber eine Hemmung der Gärung ein, welche zum Teil auf eine Verlängerung der Induktionsperiode zurückzuführen ist. In 0,2 bzw. 0,3, 0,4 und 0,5 M Bernsteinsäure-Succinat-Puffer hatten sich mithin nach 240 Min. nur 21,0 bzw. 5,3, 1,9 und 1,3 ml Kohlendioxyd entwickelt, während in dest. Wasser bzw. 0,1 M Puffer in der gleichen Zeitspanne 30,2 bzw. 44,3 ml gebildet worden waren. Unter anaeroben Bedingungen konnte bei sämtlichen Konzentrationen des Puffers gegenüber dest. Wasser eine Gärungshemmung festgestellt werden. Die nach 240 Min. in 0,1 bzw. 0,2, 0,3, 0,4 und 0,5 M Bernsteinsäure-Succinat-Lösung anaerob entwickelten Kohlendioxydvolumina betrugen nur 23,4 bzw. 4,7, 1,7, 0,8 und 0,5 ml gegenüber 30,3 ml in dest. Wasser.

Die in Abb. 4 dargestellten Resultate werden durch die in Abb. 5 aufgeführten Ergebnisse im Prinzip bestätigt. Demgemäss verläuft die Gärung in dest. Wasser in zwei Phasen, deren durchschnittliche Gärgeschwindigkeit in Luft 2,9 bzw. 2,0 und in $O_2$-freiem Stickstoff 3,0 bzw. 1,9 ml pro 15 Min. beträgt. Der Übergang der Phasen ineinander findet in der Zeitspanne von 105 bis 150 Min. statt, wo die Gärgeschwindigkeit unter aeroben bzw. anaeroben Bedingungen verhältnismässig rasch von 2,8 auf 2,1 bzw. von 3,2 auf 2,1 ml pro 15 Min. sinkt. In Luft bzw. Stickstoff hatten sich nach 105 Min. 20,3 bzw. 20,4 und nach 150 Min. 28,2 bzw. 27,9 ml Kohlendioxyd entwickelt. Im Durchschnitt entspricht dies 24,3 bzw. 24,2 ml. In den Gärungen, welche zu Ende geführt wurden, erhielten wir im Mittel 49,3 ml ($\delta = 0,8$). Die Gärkurven biegen demnach um den Punkt herum ab, wo approximativ die Hälfte der zugesetzten Zuckermenge verbraucht worden ist. Ferner ist ersichtlich, dass unter aeroben Bedingungen die in 0,04 bzw. 0,08 und 0,12 M Bernsteinsäure-Succinat-Puffer aufgeschwemmten Hefezellen eine wesentlich grössere Gärfähigkeit aufweisen als die in dest. Wasser suspendierten Zellen. Die nach 150 Min. in den Pufferlösungen entwickelten Kohlendioxydvolumina betrugen 35,9 bzw. 36,1 und 34,8

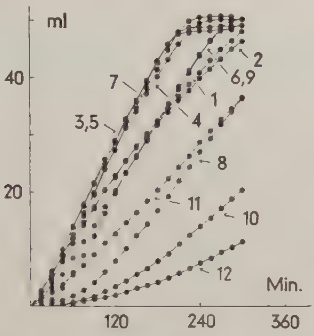


Abb. 5. Aerobe und anaerobe Vergärung der Glucose in dest. Wasser und in Bernsteinsäure-Kaliumsuccinat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 17 Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Dest. Wasser: | | 1 | 2 |
| Bernsteinsäure-Kaliumsuccinat | 0,04 M: | 3 | 4 |
| „ | 0,08 M: | 5 | 6 |
| „ | 0,12 M: | 7 | 8 |
| „ | 0,16 M: | 9 | 10 |
| „ | 0,20 M: | 11 | 12 |

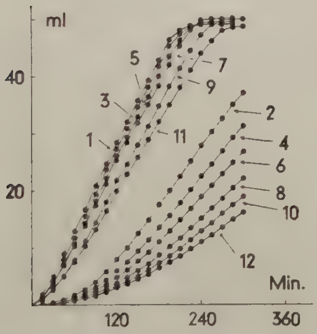


Abb. 6. Aerobe und anaerobe Vergärung der Glucose in Bernsteinsäure-Kaliumsuccinat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Bernsteinsäure-Kaliumsuccinat | 0,11 M: | 1 | 2 |
| „ | 0,12 M: | 3 | 4 |
| „ | 0,13 M: | 5 | 6 |
| „ | 0,14 M: | 7 | 8 |
| „ | 0,15 M: | 9 | 10 |
| „ | 0,16 M: | 11 | 12 |

ml gegenüber 28,2 ml in dest. Wasser. Unter anaeroben Bedingungen konnte nur bei der in 0,04 M Puffer aufgeschwemmten Hefe eine kräftige Stimulierung des Gärvermögens festgestellt werden. In diesem Fall waren nach 150 Min. 34,2 ml Kohlendioxyd gebildet worden. Bei der Hefesuspension in 0,16 M Bernsteinsäure-Succinat-Lösung lässt sich unter aeroben und bei derjenigen in 0,08 M Puffer unter anaeroben Bedingungen im früheren Gärverlauf eine deutliche Hemmung und im späteren Verlauf eine Stimulierung des Zuckerumsatzes gegenüber den Zellen in dest. Wasser feststellen, wobei der Schnittpunkt der Kurven bei 180 Min. Gärzeit liegt. Die in den erwähnten Systemen in diesem Zeitpunkt gemessenen Kohlendioxydmengen betrugen der Reihenfolge nach 31,9 bzw. 32,4, 32,6 (Luft) und 32,0 (Stickstoff) ml. In 0,12 und 0,16 sowie 0,2 M Bernsteinsäure-Succinat-Lösung tritt unter anaeroben und bei der letzten Konzentration auch unter aeroben Bedingungen im Vergleich zum dest. Wasser eine starke Hemmung des Gärvermögens der Hefezellen ein, indem nach 150 Min. nur 12,4 und 5,6 bzw. 3,1 und 16,5 ml Kohlendioxyd gebildet worden waren (dest. Wasser: Anaerob 27,9 und aerob 28,2 ml). Um die Konzentrationsabhängigkeit der Bernsteinsäure-Succinat-Wirkung auf die Gärfähigkeit des aus jungen Kulturen geernteten Hefematerials einleuchtend zu machen, sei schliesslich zusammenfassend erwähnt, dass sich in 0,04 bzw. 0,08, 0,12, 0,16 und 0,2 M Puffer nach 150 Min. unter aeroben Bedingungen 35,9 bzw. 36,1, 34,8, 26,2 und 16,5 ml Kohlendioxyd entwickelt hatten, während für die anaerob durchgeführten Parallelgärungen in der gleichen Zeitspanne 34,2 bzw. 26,5, 12,4, 5,6 und 3,1 ml ermittelt wurden.

Dass die aus jungen Kulturen gewonnene Hefe bereits auf sehr kleine Aenderungen in der Konzentration des Bernsteinsäure-Succinat-Puffers bei pH 4,9 reagiert, geht aus Abb. 6 hervor. In diesem Versuch betrugen die in 0,11 bzw. 0,12, 0,13, 0,14, 0,15 und 0,16 M Pufferlösung nach 180 Min. in Luft entwickelten Kohlendioxydmengen 42,9 bzw. 41,8, 40,1, 38,5, 34,9 und 32,1 ml. In $O_2$-freiem Stickstoff konnten zur gleichen Zeit nur 17,5 bzw. 13,5, 11,0, 8,7, 7,3 und 6,4 ml Gärgas festgestellt werden. Im geprüften Bereich lassen sich also Aenderungen der Molarität von Bernsteinsäure-Succinat-Lösungen in Stufen von 0,01 mit Hilfe der volumetrischen Messung der aeroben und anaeroben Kohlendioxydproduktion durch Zellen aus jungen Kulturen der Kulturweinhefe ,,Fendant'' glatt und mit bemerkenswerter Präzision nachweisen.

### Die Konzentrationsabhängigkeit der Wirkung von Citronensäure-Natriumcitrat-, Fumarsäure-Natriumfumarat-, DL-Aepfelsäure-Natriummalat-, D-Weinsäure-Natriumtartrat- und Essigsäure-Natriumacetat-Lösungen auf den aeroben und anaeroben Glucoseumsatz in Suspensionen von Hefezellen aus jungen Kulturen.

Es wurden drei Versuche durchgeführt und zwar mit Zellmaterial aus 16½ bzw. 16½ und 15½ Std. alten Hefekulturen. Im ersten Experiment wurden 0,1, 0,3 und 0,5 M Citronensäure-Natriumcitrat- bzw. Fumarsäure-Natriumfumarat-Lösungen, im zweiten Experiment 0,1, 0,3 und 0,5 M DL-Aepfelsäure-Natriummalat- bzw. D-Weinsäure-Natriumtartrat-Lösungen und im dritten Experiment 0,05, 0,1, 0,15, 0,3 und 0,5 M Essigsäure-Natriumacetat-Lösungen sowie dest. Wasser als Suspensionsmittel geprüft. Der pH-Wert der Pufferlösungen betrug durchgehend 4,9. Die Hefe wurde in sämtlichen Fällen zunächst 2mal in dest. Wasser und 1mal in dem zu prüfenden Puffersystem gewaschen, wobei in den beiden ersten Versuchen 0,1 M Lösungen (Citronensäure-Citrat bzw. Fumarsäure-Fumarat, Aepfelsäure-Malat bzw. Weinsäure-Tartrat) und im letzten Versuch eine 0,05 M Lösung (Essigsäure-Acetat) zur Verwendung kamen. Im letzten Experiment wurde die Hefe sodann wiederum 1mal in dest. Wasser gewaschen. Zur Ermittlung der Kohlendioxydproduktion wurden Zellsuspensionen in dest. Wasser hergestellt und diese mit Pufferlösung der doppelten für den Gärversuch gewünschten Molarität im Volumenverhältnis 1 : 1 versetzt. Das Schütteln der Suspensionen in Luft bzw. $O_2$-freiem Stickstoff dauerte 120 Min.

Der Verlauf der in Citronensäure-Citrat-Lösungen durchgeführten Gärungen geht aus Abb. 7 hervor. Es ist ersichtlich, dass mit wachsender Konzentration dieses Puffers eine Gärungshemmung eintritt und zwar sowohl in Luft als auch in $O_2$-freiem Stickstoff. In 0,1 bzw. 0,3 und 0,5 M Pufferlösung betragen mithin die nach 150 Min. entwickelten Kohlendioxydvolumina 32,0 bzw. 26,4 und 12,0 ml unter aeroben und 31,5 bzw. 26,5 und 11,8 ml unter anaeroben Bedingungen. Ferner ist ersichtlich, dass bei den zwei niedrigeren Pufferkonzentrationen die Vergärung des zugesetzten Zuckers längs praktisch identischen Kurven verläuft, unabhängig davon, ob sie aerob oder anaerob durchgeführt wird. Erst in der 0,5 M Citronensäure-Citrat-Lösung konnte in $O_2$-freiem Stickstoff eine grössere Gärgeschwindigkeit festgestellt werden als in Luft und zwar im späteren Verlauf

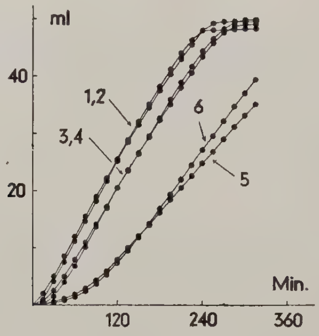


Abb. 7. Aerobe und anaerobe Vergärung der Glucose in Citronensäure-Natriumcitrat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Citronensäure-Natriumcitrat | 0,1 M: | 1 | 2 |
| „ | 0,3 M: | 3 | 4 |
| „ | 0,5 M: | 5 | 6 |

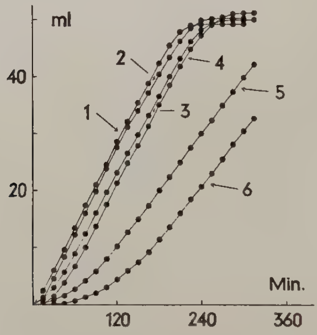


Abb. 8. Aerobe und anaerobe Vergärung der Glucose in Fumarsäure-Natriumfumarat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Fumarsäure-Natriumfumarat | 0,1 M: | 1 | 2 |
| „ | 0,3 M: | 3 | 4 |
| „ | 0,5 M: | 5 | 6 |

der Gärung (nach 165 Min.). Dies stellt eine gewisse Diskrepanz gegenüber den Ergebnissen früherer Versuche dar, in welchen sich ja unter anaeroben Bedingungen in 0,16 M Citronensäure-Citrat-Puffer durchgehend eine zwar verhältnismässig kleine aber doch deutliche Gärungsförderung feststellen liess (vgl. oben). Wir werden unten erörtern, ob diese Diskrepanz auf Unterschiede in der Vorbehandlung des Hefematerials zurückgeführt werden kann.

Die bei der Vergärung der Glucose in Fumarsäure-Fumarat-Puffer verschiedener Konzentrationen erhaltenen Gärkurven sind in Abb. 8 aufgeführt. Es ist ersichtlich, dass auch in diesem Puffersystem bei steigender Konzentration eine Gärungshemmung einsetzt. In 0,1 bzw. 0,3 und 0,5 M Lösung hatten sich mithin unter aeroben Bedingungen nach 150 Min. 35,4 bzw. 29,8 und 14,9 ml Kohlendioxyd gebildet. In $O_2$-freiem Stickstoff konnten nach der gleichen Zeitspanne 34,0 bzw. 27,9 und 7,4 ml Gärgas festgestellt werden. In der annähernd geradlinigen Gärphase ist die Gärgeschwindigkeit bei den zwei niedrigeren Pufferkonzentrationen approximativ gleich, und zwar sowohl unter aeroben als auch unter anaeroben Bedingungen. Die geringe in $O_2$-freiem Stickstoff auftretende Gärungshemmung ist in diesen Fällen ebenso wie die konzentrationsbedingte Hemmung auf eine Verlängerung der Induktionsperiode zurückzuführen. Dagegen setzt in der 0,5 M Fumarsäure-Fumarat-Lösung eine wesentliche Gärungshemmung ein, welche im Prinzip mit derjenigen im Bernsteinsäure-Succinat-Puffer übereinstimmt, indem sie unter anaeroben Bedingungen viel stärker ist als in Luft.

Wie aus Abb. 9 hervorgeht, verhalten sich die mit Aepfelsäure-Malat-Puffer verschiedener Konzentrationen bereiteten Hefesuspen-

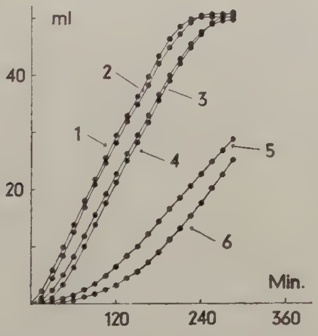


Abb. 9. Aerobe und anaerobe Vergärung der Glucose in DL-Aepfelsäure-Natriummalat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | Aerob | Anaerob |
|---|---|---|
| DL-Aepfelsäure-Natriummalat 0,1 M: | 1 | 2 |
| ,, 0,3 M: | 3 | 4 |
| ,, 0,5 M: | 5 | 6 |

sionen ähnlich wie diejenigen in Fumarsäure-Fumarat-Lösung. Bei steigender Konzentration tritt sowohl aerob als auch anaerob eine Gärungshemmung auf. In 0,1 bzw. 0,3 und 0,5 M Aepfelsäure-Malat-Puffer betrugen die nach 150 Min. entwickelten Kohlendioxydmengen 36,2 bzw. 29,4 und 10,0 ml in Luft und 34,8 bzw. 28,2 und 5,6 ml in $O_2$-freiem Stickstoff. Im approximativ linearen Abschnitt der Gärkurven ist die Geschwindigkeit der Kohlendioxydbildung bei den zwei niedrigeren Pufferkonzentrationen praktisch gleich und dies unabhängig davon, ob die Gärung aerob oder anaerob durchgeführt wird. Die sehr kleinen durch die An- bzw. Abwesenheit des molekularen gasförmigen Sauerstoffs bedingten Unterschiede in der Gärgeschwindigkeit, welche bei den in 0,1 und 0,3 M Fumarsäure-Fumarat-Lösungen aufgeschwemmten Hefezellen vorliegen, sind gleich wie die grösseren konzentrationsbedingten Unterschiede auf eine Verlängerung der Induktionsperiode zurückzuführen. Dagegen ist die in 0,5 M Lösung dieses Puffersystems auftretende Gärungshemmung prinzipiell vom gleichen Typus wie diejenige, welche von Bernsteinsäure-Succinat-Lösungen hervorgerufen wird, indem sie in $O_2$-freiem Stickstoff wesentlich grösser ist als in Luft.

Die in Abb. 7, 8 und 9 dargestellten Versuchsergebnisse unterscheiden sich somit von den in Abb. 1, 2 und 3 aufgeführten Resultaten bezüglich der Abhängigkeit des Gärvermögens des aus jungen „Fendant"-Kulturen gewonnenen Zellmaterials von der Ab- bzw. Anwesenheit des molekularen gasförmigen Sauerstoffs, und zwar gilt dies besonders für die in Fumarsäure-Fumarat- und Aepfelsäure-Malat-Lösungen hergestellten Hefesuspensionen, aber auch für diejenigen, welche in Citronensäure-Citrat-Puffer bereitet wurden. Während in den Versuchen mit 0,16 M Lösungen der erwähnten Puffersysteme (Abb. 1, 2 und 3) eine mehr oder weniger augenfällige Gärungsförderung in $O_2$-freiem Stickstoff nachgewiesen werden konnte, lässt sich in den Experimenten mit 0,1 und 0,3 M Lösungen (Abb. 7, 8 und 9) entweder keine Wirkung (Citronensäure-Citrat) oder eine minimale Förderung in Luft (Fumarsäure-Fumarat und Aepfelsäure-Malat) feststellen, wobei diese auf eine geringe Verkürzung der Induktionszeit zurückzuführen ist. Nun liegen zwischen den Experimenten, deren Resultate aus Abb. 1, 2 und 3 hervorgehen, und denjenigen, deren Ergebnisse aus Abb. 7, 8 und 9 ersichtlich sind, wesentliche Unterschiede in der Vorbehandlung des Hefematerials vor, indem beim Waschen der Hefe in den ersten Versuchen 0,16 M Säure-Kaliumsalz-Lösungen und in den letzten Versuchen 0,1 M Säure-Natriumsalz-Lösungen zur Verwendung kamen. Es ist durchaus möglich, dass sich die Diskrepanzen in bezug auf das aerobe und anaerobe Gärvermögen durch die erwähnten Unterschiede in den experimentellen Bedingungen erklären lassen. Zur endgültigen Lösung dieser Frage sind aber weitere Versuche unbedingt erforderlich. Zusammenfassend sei ferner nochmals erwähnt, dass sich die in 0,5 M Fumarsäure-Natriumfumarat- bzw. Aepfelsäure-Natriummalat-Puffer aufgeschwemmte Hefe ähnlich wie eine in 0,04–0,5 M Bernsteinsäure-Natriumsuccinat-Lösung suspendierte Hefe verhielt, indem die anaerobe Gärung im Vergleich zur aeroben eine starke Hemmung aufweist. Aus Abb. 8 und 9 geht aber deutlich hervor, dass diese Hemmung in den beiden ersten Pufferlösungen auf eine Verlängerung der Induktionsperiode zurückzuführen ist, während sie ja im Bernsteinsäure-Succinat-Puffer auch im approximativ geradlinigen Abschnitt der Gärkurve zum Vorschein kommt.

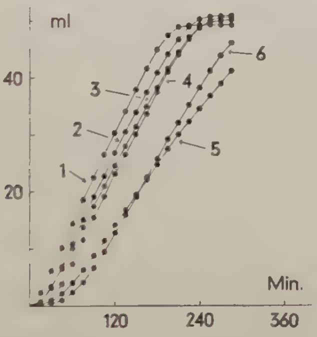


Abb. 10. Aerobe und anaerobe Vergärung der Glucose in D-Weinsäure-Natriumtartrat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 16½ Std. alten Kulturen der Weinhefe „Fendant".

| | Aerob | Anaerob |
|---|---|---|
| D-Weinsäure-Natriumtartrat 0,1 M: | 1 | 2 |
| „ 0,3 M: | 3 | 4 |
| „ 0,5 M: | 5 | 6 |

Der Gärverlauf in 0,1 bzw. 0,3 und 0,5 M Weinsäure-Natriumtartrat-Lösungen ist aus Abb. 10 ersichtlich. Die Vergärung der Glucose wird bei steigender Konzentration des Puffers sowohl unter aeroben als auch unter anaeroben Bedingungen gehemmt. Bei den beiden niedrigeren Konzentrationen zeigte die in $O_2$-freiem Stickstoff durchgeführte Gärung gleich wie in früheren Versuchen (Abb. 1 und 3) im Vergleich zur aeroben Gärung eine Hemmung, die aber in 0,3 M Pufferlösung äusserst gering ist. In 0,5 M Weinsäure-Tartrat-Lösung weist dagegen die anaerobe Gärung nach einer Gärdauer von 150–160 Min. gegenüber dem aeroben Zuckerumsatz eine Stimulierung auf. Bei wachsender Konzentration des Weinsäure-Tartrat-Puffers tritt somit nicht nur eine allgemeine Gärungshemmung ein, sondern auch eine Umstimmung des Gärtypus hinsichtlich der Abhängigkeit vom Luftsauerstoff.

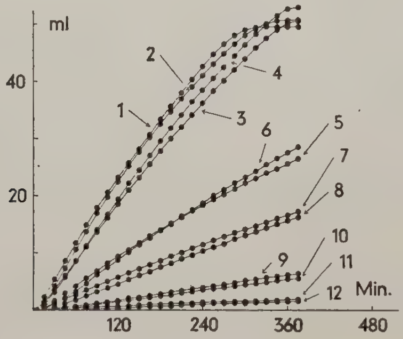


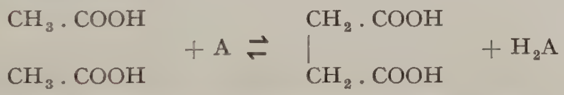
Abb. 11. Aerobe und anaerobe Vergärung der Glucose in dest. Wasser und in Essigsäure-Natriumacetat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 15½ Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Dest. Wasser: | | 1 | 2 |
| Essigsäure-Natriumacetat | 0,05 M: | 3 | 4 |
| „ | 0,10 M: | 5 | 6 |
| „ | 0,15 M: | 7 | 8 |
| „ | 0,30 M: | 9 | 10 |
| „ | 0,50 M: | 11 | 12 |

Die in Essigsäure-Natriumacetat-Puffer verschiedener Konzentrationen erhaltenen Gärkurven sind in Abb. 11 aufgeführt. Es ist ersichtlich, dass sämtliche in dieser Pufferlösung durchgeführten Gärungen im Vergleich zum parallelen Zuckerumsatz in dest. Wasser eine Hemmung aufweisen, welche bei steigender Konzentration des Puffers stark zunimmt. Nach 180 Min. hatten sich unter aeroben Bedingungen in dest. Wasser bzw. 0,05, 0,1, 0,15, 0,3 und 0,5 M Essigsäure-Natriumacetat-Lösung 33,3 bzw. 27,8, 14,0, 8,6, 2,8 und 1,0 ml Kohlendioxyd entwickelt, während in Stickstoffatmosphäre 32,5 bzw. 29,5, 13,9, 7,5 2,4 und 0,7 ml festgestellt werden konnten. Es geht aus diesen Werten ferner hervor, dass die Unterschiede der

Gärgeschwindigkeiten zwischen den aerob und anaerob durchgeführten Gärungen sehr gering sind. Der Verlauf der Gärungskurven lässt aber erkennen, dass auch bei der in Essigsäure-Natriumacetat-Lösungen aufgeschwemmten Hefe mit wachsender Konzentration des Puffers eine Tendenz zur Umstimmung des Gärtypus vorliegt, indem bei der kleinsten Konzentration (0,05 M) der anaerobe und bei den drei höchsten Konzentrationen (0,15, 0,3 und 0,5 M) der aerobe Zuckerumsatz ein wenig gefördert zu sein scheint. Der Wendepunkt liegt bei dem untersuchten Hefematerial im Konzentrationsbereich von etwa 0,1 M, wo die beiden Gärungskurven sich nach einer Versuchszeit von 200 Min. kreuzen.

Im Hinblick auf die Thunberg-Wieland-Kondensation und ihre Reversibilität (siehe Oppenheimer und Stern (29) S. 243, 250; Slade und Werkman (35)) sei besonders hervorgehoben, dass bei der in Essigsäure-Natriumacetat-Puffer gewaschenen und suspendierten Hefe kein $O_2$-Effekt auf die Gärgeschwindigkeit zum Vorschein kommt, wie wir ihn bei jungem Hefematerial in Bernsteinsäure-Succinat-Lösungen haben feststellen können. Dies bedeutet, dass die aus jungen Kulturen stammende „Fendant"–Hefe unfähig ist, während des Schüttelns der Suspensionen in Luft oder $O_2$-freiem Stickstoff die Thunberg-Wieland-Kondensation in nachweisbarem Ausmasse zu bewirken:



$$\begin{matrix} CH_3 . COOH \\ \\ CH_3 . COOH \end{matrix} + A \rightleftarrows \begin{matrix} CH_2 . COOH \\ | \\ CH_2 . COOH \end{matrix} + H_2A$$

Aus Abb. 4, 5 und 6 geht hervor, dass der beschriebene Bernsteinsäure-Succinat-Effekt in 0,04 M Lösung des Puffers kaum sichtbar ist und erst im Konzentrationsbereich 0,08–0,1 M deutlich zum Vorschein kommt. Ein Effekt wäre somit nur im Falle eines beträchtlichen Umsatzes der Essigsäure nach Thunberg und Wieland zu erwarten. Ferner ist zu berücksichtigen, dass die von der Hefe etwa gebildete Bernsteinsäure nicht unbedingt in der gleichen Weise an die Zellstrukturen gebunden oder in diesen lokalisiert wird wie die von aussen in der Form einer Pufferlösung vom pH 4,9 zugesetzte Säure. Die hier gestreifte Frage ist von grosser theoretischer und praktischer Bedeutung, da die Ergebnisse unserer Untersuchungen (40, 41, 42, 43, 44) darauf hindeuten, dass das Bernsteinsäure-Succinat-System unter Umständen als ein Regulator der Geschwindigkeit der aeroben und anaeroben alkoholischen Gärung bei den Mikroorganismen auftreten kann.

### Die Konzentrationsabhängigkeit der Wirkung des Bernsteinsäure-Kaliumsuccinat-Puffers auf den aeroben und anaeroben Glucoseumsatz in Suspensionen von Hefezellen aus alten Kulturen.

Wie bereits erwähnt, konnte der Bernsteinsäure-Succinat-Effekt auf die Vergärung der Glucose bei ruhendem Zellmaterial aus alten Kulturen der Weinheferasse „Fendant" nicht oder nur in geringem Ausmasse festgestellt werden (40, 41, 42, 43). In den betreffenden Versuchen wurde stets mit einer 0,16 M Pufferlösung gearbeitet. Es interessierte uns nun, ob sich ein Effekt bei anderen Konzentrationen des Bernsteinsäure-Succinat-Systems nachweisen lassen könnte. Deshalb wurde ein Experiment mit Hefe aus einer 69 Std. alten Kultur ausgeführt. Dieses Zellmaterial wurde zunächst 2mal in dest. Wasser und 1mal in 0,16 M Bernsteinsäure-Kaliumsuccinat-Lösung und sodann aufs neue 1mal in dest. Wasser gewaschen. Es wurde in dest. Wasser aufgeschwemmt. Die Hefesuspension wurde so im Volumenverhältnis 1 : 1 mit Pufferlösungen versetzt, deren Molaritäten doppelt so gross wie die im Gärversuch zu prüfenden Konzentrationen waren. Vor dem Zusetzen der Glucoselösungen, welche mit den betreffenden Pufferlösungen bereitet worden waren, wurden die Hefesuspensionen während 120 Min. in ruhender Luft bzw. strömendem $O_2$-freiem Stickstoff geschüttelt. Geprüft wurden 0,1, 0,2, 0,3, 0,4 und 0,5 M Lösungen vom pH 4,9. Mit dem gleichen Hefematerial wurden parallele Gärungen in dest. Wasser durchgeführt. Die Ergebnisse dieses Versuches sind in Abb. 12 dargestellt. Es ist ersichtlich, dass bei der in Bernsteinsäure-Succinat-Lösungen aufgeschwemmten Hefe mit steigender Konzentration des Puffers eine Hemmung der aeroben und anaeroben Gärung eintritt. In 0,1 bzw. 0,2, 0,3, 0,4 und 0,5 M Pufferlösung betrugen die aerob entwickelten Kohlendioxydvolumina 28,1 bzw. 27,2, 26,4, 22,6 und 17,4 ml nach 180 Min. und 39,2 bzw. 37,7 36,8, 32,3 und 25,4 ml nach 240 Min. Unter anaeroben Bedingungen konnten 31,1 bzw. 28,9, 25,9, 22,5 und 18,6 ml nach 180 Min. und 41,6 bzw. 39,2, 35,4, 31,4 und 26,1 ml nach 240 Min. festgestellt werden. Diese Werte zeigen, dass die bei altem Hefematerial mit steigender Pufferkonzentration einsetzende Gärungshemmung äusserst klein ist im Vergleich zu der Hemmung, welche bei Zellmaterial aus jungen Kulturen hervorgerufen wird (Abb. 4 und 5). Es geht ferner hervor, dass bei der alten Hefe nicht einmal bei den höchsten geprüften Pufferkonzentrationen ein Bernsteinsäure-Succinat-Effekt zum Vorschein kommt, wie wir ihn bei jungem Zellmaterial haben nachweisen können. In dest. Wasser hatten sich in Luft bzw. in $O_2$-freiem Stickstoff nach 180 Min. 25,0 bzw. 27,1 ml und nach 240 Min. 33,6 bzw. 35,1 ml Kohlendioxyd entwickelt. Dies bedeutet, dass die Gärung bei altem Zellmaterial in 0,1 und 0,2 M Bernsteinsäure-Kaliumsuccinat-Lösung sowohl aerob als auch anaerob und in 0,3 M Pufferlösung aerob eine Stimulierung gegenüber den in dest. Wasser parallel durchgeführten Gärungen aufweist. In diesem Zusammenhang sei daran erinnert, dass bei Hefematerial aus jungen Kulturen nur in 0,04 M Bernsteinsäure-Succinat-Lösung sowohl aerob als auch anaerob und in 0,08, 0,1 und 0,12 M Puffer aerob eine deutliche Stimulierung der Gärung im Vergleich zum parallelen Zuckerumsatz in dest. Wasser festgestellt werden konnte (Abb. 4 und 5). Auch in dieser Beziehung bestehen also wesentliche Unterschiede zwischen den aus alten und jungen Hefekulturen gewonnenen Zellen in der Empfindlichkeit gegen das Bernsteinsäure-Succinat-System.

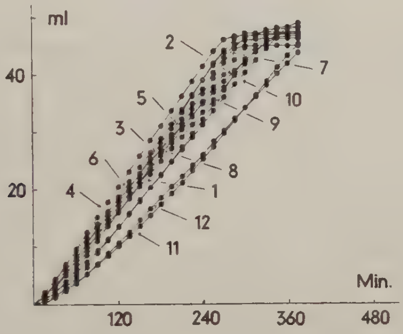


Abb. 12. Aerobe und anaerobe Vergärung der Glucose in dest. Wasser und in Bernsteinsäure-Kaliumsuccinat-Puffer verschiedener Konzentrationen bei pH 4,9 durch ruhende Zellen aus 69 Std. alten Kulturen der Weinhefe „Fendant".

| | | Aerob | Anaerob |
|---|---|---|---|
| Dest. Wasser | | 1 | 2 |
| Bernsteinsäure-Kaliumsuccinat | 0,1 M: | 3 | 4 |
| ,, | 0,2 M: | 5 | 6 |
| ,, | 0,3 M: | 7 | 8 |
| ,, | 0,4 M: | 9 | 10 |
| ,, | 0,5 M: | 11 | 12 |

## Diskussion und Schlussfolgerungen.

In der vorliegenden Arbeit konnte bestätigt werden, dass das Bernsteinsäure-Succinat-System bei ,,ruhendem'' Zellmaterial aus jungen Hefekulturen unter anaeroben Bedingungen eine starke Hemmung der alkoholischen Gärung hervorruft (Abb. 2, 3, 4, 5 und 6), während dies bei dem aus alten Kulturen gewonnenen Zellmaterial nicht der Fall ist (Abb. 12).

Es wurde ferner festgestellt, dass der erwähnte Effekt nicht nur in Bernsteinsäure-Natriumsuccinat-Puffer (40, 41, 42, 43, 44) sondern auch in Bernsteinsäure-Kaliumsuccinat-Lösung auftritt. Es scheint somit ausgeschlossen, dass er auf das Konzentrationsverhältnis K/Na im Sinne einer ungünstigen Beeinflussung der Reversibilität der durch Phosphoenoltransphosphorylase katalysierten Dephosphorylierung der Enolbrenztraubensäurephosphorsäure zurückzuführen ist.

Die in Bernsteinsäure-Succinat-Puffer unter anaeroben Bedingungen bei ,,ruhenden'' Zellen aus jungen Kulturen auftretende Gärungshemmung ist reversibel, indem sie durch Zusatz von gasförmigem molekularem Sauerstoff vor dem Beimischen des Zuckers aufgehoben wird. Im Bereich von 0,005–10 à 20 Volumen-% Sauerstoff steigt das Ausmass der Reaktivierung des Zymasesystems mit wachsender $O_2$-Konzentration (40, 41, 42, 43, 44). Der Effekt könnte als ein Beispiel der ,,Erscheinung maskierter Enzyme'' (,,phenomenon of the masked enzymes'' nach Kluyver (12), S. 117) betrachtet werden, wobei allerdings berücksichtigt werden soll, dass die ,,Demaskierung'' bereits in den intakten Zellen fast momentan stattfindet und nicht erst nach einer beträchtlichen Induktionsperiode (,,lag period'') oder nachdem die Zellstrukturen durch Mazeration zerstört worden sind.

Es sei daran erinnert, dass die alkoholische Gärung der Hefen nach klassischen Begriffen als ein anoxydativer oder anoxybiontischer Vorgang, d.h. als ein ohne $O_2$-Aufnahme verlaufender Prozess, betrachtet wird, der sich glatt unter anaeroben Bedingungen, d.h. in vollständiger Abwesenheit des gasförmigen molekularen Sauerstoffs, abspielen kann. Bei den in 0,16 M Bernsteinsäure-Succinat-Puffer durchgeführten Versuchen trifft dies nur im Falle des alten Hefematerials zu, während die aus jungen Kulturen stammenden Zellen in bezug auf die alkoholische Gärung als mikroaerophil bis aerob bezeichnet werden müssen, indem sich der erwähnte Prozess mit normaler Geschwindigkeit nur in Gegenwart minimaler bis grosser $O_2$-Mengen abspielt. Die Tatsache, dass eine gärungsfördernde Wirkung bereits in Anwesenheit von 0,005 Volumen-% Sauerstoff in der Gasphase der Gärkolben (von etwa 50 ml Inhalt), d.h. durch 0,0025–0,003 ml oder 2,5–3 $\mu$l $O_2$ pro 300 mg feuchte Hefe oder ca. 60 mg Hefetrockensubstanz, hervorgerufen wird, deutet darauf hin, dass diese Wirkung über eine Aktivierung einer oder mehrerer Komponenten der im jungen Zellmaterial vorhandenen Zymase oder der damit verknüpften Fermentsysteme stattfindet, wobei es sich um eine Dehydrierung, eine Anlagerung von Sauerstoff oder einen Valenzwechsel (Elektronenabgabe) handeln könnte. Wie wir in einem separaten Artikel ausführen werden, tritt die Aktivierung nur unter der Voraussetzung ein, dass der $O_2$-Zusatz vor dem Beimischen der Glucose erfolgt. Die Gärgeschwindigkeit wird somit bei ein und der gleichen Bernsteinsäure-Succinat-Konzentration durch die im Augenblick des Zuckerzusatzes herrschende $O_2$-Konzentration festgelegt. Dies deutet darauf hin, dass bei der im Laufe der Begasung mit $O_2$-freiem Stickstoff, Argon, Wasserstoff oder Kohlendioxyd eintretenden Gärungshemmung irgend welche Zentren in den Zellstrukturen oder aktiven zur Substratbindung befähigten Bezirken der Enzymoberfläche dermassen verändert werden, dass der Enzym-Zucker-Komplex nicht gebildet werden kann. Das Ausmass dieser Blockierung, d.h. die Anzahl inaktivierter Zentren, ist bei konstantem pH einerseits von der $O_2$-Konzentration (44), andererseits von der Stärke der Bernsteinsäure-Succinat-Lösung abhängig (Abb. 4, 5 und 6). Bei ein und demselben pH-Wert kann die gleiche Vergrösserung der Gärungshemmung einerseits durch Erhöhung der Bernsteinsäure-Succinat-Konzentration bei konstantem $O_2$-Partialdruck, andererseits durch Erniedrigung des $O_2$-Partialdrucks bei konstanter Konzentration des Puffers bewirkt werden. Das Bernsteinsäure-Succinat-System regelt bei jungem Hefematerial den Charakter der alkoholischen Gärung hinsichtlich ihrer Empfindlichkeit gegen den freien molekularen Sauerstoff. In verdünnten Bernsteinsäure-Succinat-Lösungen (Konzentration $\leqq$ 0,04 M) ist die Gärgeschwindigkeit im Bereich von 0 bis 10 à 20 Volumen-% Sauerstoff unabhängig von der $O_2$-Konzentration (Abb. 5). Bei mittleren Konzentrationen dieses Puffers verläuft die Gärung bei geringen $O_2$-Konzentrationen mit der gleichen oder einer etwas kleineren Geschwindigkeit als in Luft und kann somit als mikroaerophil bis aerob bezeichnet werden. Bei höheren Bernsteinsäure-Succinat-Konzentrationen kann nur unter aeroben Bedingungen (10–20 Volumen-% $O_2$ in der Gasphase der Gärkolben im Augenblick des Zuckerzusatzes) eine normale Gärgeschwindigkeit festgestellt werden (Abb. 4, 5 und 6). Bei sehr hohen Konzentrationen des Puffers ($\geqq$ 0,3 M) wird die alkoholische Gärung bei jungem Hefematerial nicht nur unter anaeroben, sondern auch unter aeroben Bedingungen sehr stark bis vollständig gehemmt (Abb. 4).

Bei Zellmaterial aus alten Hefekulturen tritt wohl mit steigender Konzentration des Bernsteinsäure-Succinat-Puffers (0,1–0,5 M Lösungen) eine Gärungshemmung ein. Diese ist aber im Vergleich zur Hemmung bei der aus jungen Kulturen stammenden Hefe sehr gering (Abb. 12). Im Falle des alten Hefematerials bleibt der Bernsteinsäure-Succinat-Effekt nicht nur bei kleinen, sondern auch bei hohen Konzentrationen des Puffers vollständig oder fast vollständig aus (40, 41, 42, 43; Abb. 12). Bei diesem Zellmaterial kann also dem Bernsteinsäure-Succinat-System keine Bedeutung als Regulator der Aerobiose bzw. Anaerobiose für die alkoholische Gärung beigemessen werden.

Aus den obigen Ausführungen geht hervor, dass die von RUNNSTRÖM u. Mitarb. (33, 34), BRANDT (5, 6, 7) sowie SUOMALAINEN und OURA (37) hervorgehobene Indifferenz des Bernsteinsäure-Succinat-Puffers nur für die aus alten oder verhältnismässig „reifen" Kulturen gewonnenen Hefezellen bestätigt werden konnte. Für junges Zellmaterial kann diese Pufferlösung nur bei grosser Verdünnung als „indifferent" bezeichnet werden. Bei gebräuchlichen Konzentrationen greift sie dagegen ausschlaggebend in die alkoholische Gärung, d.h. in einen der energieliefernden Prozesse der Hefe, ein. Dieser Erscheinung muss besonders in streng anaeroben Systemen grösste Bedeutung beigemessen werden, da unter diesen Bedingungen die alkoholische Gärung wohl alleinige Quelle der Energie ist, welche zur Aufrechterhaltung der Zellstrukturen sowie zur Zellsubstanzsynthese und Zellvermehrung unerlässlich („essential") ist.

Unsere Befunde zeigen ferner, dass in Untersuchungen über die physiologische Bedeutung verschiedener Faktoren und Prozesse die Wahl des Zellmaterials mit grösster Sorgfalt und unter Berücksichtigung der im Laufe der Entwicklung der betreffenden Mikroorganismenkulturen stattfindenden Veränderungen der Zellpopulationen in physiologischer und biochemischer Hinsicht geschehen muss.

Weiterhin ist aus unseren Versuchsergebnissen zu sehen, dass der

Effekt des Bernsteinsäure-Succinat-Puffers für dieses System höchst spezifisch ist. Von den geprüften Säuren sind bekanntlich die Essigsäure, Fumarsäure, Aepfelsäure und Citronensäure über die Reaktionen der von THUNBERG und WIELAND, SZENT-GYÖRGYI, WOOD und WERKMAN, MARTIUS und KNOOP, KREBS sowie LIPMANN und zahlreichen anderen Forschern entwickelten Mono-, Di- und Tricarbonsäure-Mechanismen mit der Bernsteinsäure verknüpft. Aus den kürzlich von LA RIVIÈRE (15, 16) sowie KRAMPITZ und LYNEN (13) veröffentlichten Mitteilungen geht hervor, dass auch die D-Weinsäure im Stoffwechsel bestimmter Mikroorganismen via Oxalessigsäure mit den Komponenten der erwähnten Mechanismen in Verbindung steht. Auch wenn die von uns geprüften Säuren als solche eine Wirkung vom Typus des Bernsteinsäure-Succinat-Effekts nicht hervorzurufen vermögen, hätten wir doch einen Einfluss auf die alkoholische Gärung erwarten können, insofern diese Säuren im Laufe des Schüttelns der Hefesuspensionen in Luft oder $O_2$-freiem Stickstoff mit genügender Geschwindigkeit in Bernsteinsäure umgewandelt werden. Unsere Versuchsergebnisse zeigen nun eindeutig, dass die Systeme Citronensäure-Citrat (Abb. 1, 3 und 7), DL-Aepfelsäure-Malat (Abb. 1, 2 und 9), Fumarsäure-Fumarat (Abb. 2 und 8) und Essigsäure-Acetat (Abb. 11) im Konzentrationsbereich 0,1–0,3 M beim pH 4,9 unfähig sind, den für den Bernsteinsäure-Succinat-Puffer festgestellten Effekt zu bewirken. Im Falle der Citronensäure-Citrat- und Essigsäure-Acetat-Systeme weist auch die 0,5 M Lösung keinen Effekt auf (Abb. 7 und 11). Dagegen zeigen Fumarsäure-Fumarat und Aepfelsäure-Malat bei dieser an und für sich unphysiologischen Konzentration eine an den Bernsteinsäure-Succinat-Effekt erinnernde Wirkung (Abb. 8 und 9), die aber ganz auf eine Verlängerung der Induktionsperiode unter anaeroben Bedingungen zurückzuführen ist. Hier sei nochmals erwähnt, dass in den betreffenden Versuchen sowohl die Säure-Kaliumsalz- als auch die Säure-Natriumsalz-Kombinationen (Abb. 1, 2 und 3 bzw. Abb. 7, 8, 9 und 11) zur Verwendung kamen. Es sei ferner bemerkt, dass in gewissen Versuchen mit Fumarsäure und Aepfelsäure (Abb. 2) bzw. Citronensäure (Abb. 3) Gärungen mit dem gleichen Hefematerial parallel und simultan in Bernsteinsäure-Succinat-Puffer durchgeführt wurden und dabei den für dieses System typischen Effekt zeigten.

Im Gegensatz zu den Citronensäure-Citrat-, DL-Aepfelsäure-Malat-, Fumarsäure-Fumarat- und Essigsäure-Acetat-Systemen übt der D-Weinsäure-Tartrat-Puffer in 0,1 und 0,16 M Lösung auf die

Gärung eine Wirkung in der gleichen Richtung wie Bernsteinsäure-Succinat aus (Abb. 1, 3 und 10). Dieser Effekt ist aber sehr gering und verschwindet zudem vollständig im Konzentrationsbereich 0,3–0,5 M (Abb. 10). Die Spezifität des Bernsteinsäure-Succinat-Effekts kann somit als sehr ausgeprägt bezeichnet werden. Ueberdies geht aus unseren Versuchsergebnissen hervor, dass im Laufe des Schüttelns der Hefesuspensionen in Luft oder $O_2$-freiem Stickstoff keine nennenswerte Umwandlung der geprüften Säuren in Bernsteinsäure stattfindet. Ob der in 0,5 M Fumarsäure-Natriumfumarat- und Aepfelsäure-Natriummalat-Lösungen auftretende Effekt (Abb. 8 und 9) auf eine derartige Umwandlung zurückzuführen ist, lässt sich erst durch weitere Experimente abklären.

Die von Hägglund und Augustson (8) sowie Porchet (31) und anderen Autoren beschriebene Hemmung der alkoholischen Gärung durch Essigsäure konnte bestätigt werden (Abb. 11). Im Vergleich mit den in dest. Wasser parallel und simultan durchgeführten Gärungen setzt sie beim jungen Hefematerial bereits in 0,05 M Essigsäure-Natriumacetat-Lösungen vom pH 4,9 ein und zwar sowohl unter aeroben als auch unter anaeroben Bedingungen. In 0,1 M Lösung und bei den höheren Konzentrationen ist die Gärungshemmung ausserordentlich stark. Die Essigsäure-Acetat-Gemische nach Walpole oder Michaelis, welche als 0,1 oder 0,2 M Lösungen in biochemischen und physiologischen Versuchen häufig zur Verwendung kommen, können somit vom physiologischen Gesichtspunkt aus kaum als einwandfrei betrachtet werden.

In den Systemen Bernsteinsäure-Succinat (Abb. 4, 5, 6 und 12), Citronensäure-Citrat (Abb. 7), Fumarsäure-Fumarat (Abb. 8), DL-Aepfelsäure-Malat (Abb. 9) und D-Weinsäure-Tartrat (Abb. 10) setzen mit steigender Pufferkonzentration sowohl unter aeroben als auch unter anaeroben Bedingungen ebenfalls Gärungshemmungen ein. In 0,16 M Lösung hemmt die Bernsteinsäure wesentlich stärker als Aepfelsäure, Fumarsäure (Abb. 2), Citronensäure und Weinsäure (Abb. 3), und zwar nicht nur in $O_2$-freiem Stickstoff sondern auch in Luft. Die in den Puffersystemen der vier letzteren Säuren suspendierten Hefezellen weisen nur verhältnismässig kleine Gärungsunterschiede auf. Zusammenfassend kann gesagt werden, dass die gärungshemmende Tendenz in der Reihenfolge Aepfelsäure, Fumarsäure und Citronensäure zunimmt und dies sowohl aerob als auch anaerob. Die Weinsäure verhält sich unter aeroben Bedingungen wie die Aepfelsäure, wirkt aber unter anaeroben Bedingungen bei gewissen Konzentrationen ungünstiger als die erwähnten drei Säuren (Abb. 1, 2, 3, 7, 8, 9 und 10). Wie schon besprochen, lässt sich in D-Weinsäure-Tartrat-Lösungen mittlerer Konzentration eine Wirkung vom Typus des Bernsteinsäure-Succinat-Effekts in kleinem Ausmass nachweisen.

Von den hier untersuchten Pufferlösungen organischer Säuren stellt das Bernsteinsäure-Succinat-Gemisch hinsichtlich der alkoholischen Gärung der Hefe zweifellos das am wenigsten indifferente System dar und zwar, wie ersichtlich, u.a. deshalb, weil die durch dieses Gemisch unter anaeroben Bedingungen hervorgerufene Gärungshemmung wesentlich grösser ist als die Hemmung, welche bei der gleichen Pufferkonzentration in Anwesenheit des freien molekularen Sauerstoffs bewirkt wird. Wir verzichten vorläufig auf eine Diskussion der Bedeutung, die das aus Succinodehydrogenase, Cytochromen, Cytochromoxydase und anderen Komponenten bestehende System für diese Erscheinung haben könnte.

Es wäre verlockend, hier die Begriffe der ,,adaptiven" und ,,konstitutiven" Enzyme nach KARSTRÖM (10, 11) und der ,,induzierten" Enzyme nach MONOD (20,21; siehe ferner SPIEGELMAN und CAMPBELL (36)) zu besprechen und zwar im Lichte unserer Befunde bezüglich der spezifischen Interferenz des Bernsteinsäure-Succinat-Systems und des freien molekularen Sauerstoffs mit einer oder mehreren Komponenten des Zymasesystems in Hefezellen aus jungen Kulturen. Wir werden diese Begriffe jedoch erst in einem späteren Artikel diskutieren, wo wir die Ergebnisse von Versuchen mit bestimmten Kohlehydraten beschreiben wollen, die in verschiedenen Mengen den Hefesuspensionen vor deren Begasung mit $O_2$-freiem Stickstoff beigemischt wurden.

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## Summary.

The present paper deals with the effects produced at pH 4.9 by the buffers succinic acid-succinate, DL-malic acid-malate, fumaric acid-fumarate, citric acid-citrate, D-tartaric acid-tartrate, and

acetic acid-acetate upon the alcoholic fermentation of glucose by resting cells of the auxo-autotrophic tame wine yeast Fendant under aerobic and anaerobic conditions (air and oxygen-free nitrogen, respectively).

As regards the general growth and fermentation conditions applied in this investigation we refer to the summaries given by WIKÉN and RICHARD (39, 41, 43). Here it may only be mentioned that the yeast suspensions examined for fermentation capacity were washed by suspending and centrifuging in distilled water and, subsequently, in the buffer solution used in the fermentation experiment. In detail the washing procedure applied in the different experiments was as follows:

Fig. 1: Twice in distilled water, and once in 0.16 M solution of the respective buffer.

Fig. 2 and 3: Twice in distilled water, and thrice in 0.16 M solution of the buffer concerned.

Fig. 4, 5 and 6: Twice in distilled water, once in 0.16 M buffer, and once again in distilled water.

Fig. 7, 8, 9 and 10: Twice in distilled water, and once in 0.1 M solution of the proper buffer.

Fig. 11: Twice in distilled water, once in 0.05 M buffer, and once again in distilled water.

Fig. 12: Twice in distilled water, once in 0.16 M buffer, and once again in distilled water.

The yeast thus washed was suspended in distilled water or the buffer solutions concerned. Subsequently, the cell suspensions were shaken for 120 minutes in air (aerobic fermentation) or in streaming oxygen-free nitrogen (anaerobic fermentation) previous to the addition of sugar.

The cells from young cultures were harvested after incubation at 20°C. or 23°C. for 15½ to 17 hours, and those from old cultures after incubation at the same temperatures for 69 hours. The former yeast material corresponds to the period of cellular adjustment (= initial stationary phase + phase of accelerating growth and multiplication rate).

The results obtained in the present and some earlier experiments with cells from young cultures may be summarized as follows:

The suspensions prepared in distilled water show the same or practically the same fermentation rate in oxygen-free nitrogen as in air. Further, the fermentation curves assume a more or less broken form, the transition from the faster to the slower mode of carbon dioxide formation occurring at the stage where about half of the sugar added has been used up (Fig. 4, 5 and 11). In some experiments (cf. Fig. 4 and 5) the break in the fermentation curves is rather distinct whereas in other experiments (cf. Fig. 11) the first segment of the curves is not sharply separated from the second one, the decrease of fermentation with time thus in the latter case occurring more continuously.

The fermentation effected by the yeast suspensions in 0.04–0.12 M solutions of succinic acid-succinate (pH = 4.9) showed under aerobic conditions a strong stimulation compared to the fermentation brought about by the corresponding suspensions in distilled water (Fig. 4 and 5). Under anaerobic conditions this stimulation sets in only at lower concentrations of the buffer mentioned (0.04 M solution; Fig. 5). Further, the aerobic yeast suspensions in 0.16 M succinic acid-succinate and the anaerobic ones in 0.08 M solution of the same buffer are inhibited in the earlier stage of fermentation but stimulated in the later stage as compared to the suspensions in distilled water (Fig. 5). At higher concentrations of succinic acid-succinate the fermentation capacity of the yeast cells from young cultures is strongly inhibited, the rate of carbon dioxide formation decreasing with increasing strength of the buffer (Fig. 4, 5 and 6).

From a comparison of the rates of carbon dioxide formation observed in parallel yeast suspensions prepared with different buffer solutions it may be concluded that also fumaric acid-fumarate, DL-malic acid-malate, citric acid-citrate and D-tartaric acid-tartrate at low concentrations stimulate the fermentation in cells from young cultures (Fig. 1, 2, 3, 7, 8, 9 and 10 as compared to Fig. 4, 5 and 6) whereas in acetic acid-acetate an inhibiting effect upon the fermenting power is evident even at 0.05 M concentration.

At higher concentrations all buffers examined induce an inhibition of fermentation, the rate of carbon dioxide production decreasing considerably with increasing strength of the buffers concerned (Fig. 4–11).

The effect produced by succinic acid-succinate upon the alcoholic fermentation of cells from young yeast cultures is specific as far as the difference in inhibiting power under aerobic and anaerobic conditions is concerned, the inhibition being considerably larger in oxygen-free nitrogen, argon, hydrogen or carbon dioxide than in air or synthetic mixtures of oxygen and nitrogen (Fig. 2–6; WIKÉN and RICHARD (40–44)). A similar effect is obtained in D-tartaric acid-tartrate solution. This effect, however, is minute and disappears on increasing the concentration of the buffer (Fig. 1, 3 and 10). In fumaric acid-fumarate and DL-malic acid-malate an effect similar to that produced by succinic acid-succinate is observed only at high concentrations far beyond the physiological range (0.5 M; Fig. 1, 2, 8 and 9). In citric acid-citrate and acetic acid-acetate solutions we did not find any effect of the kind mentioned (Fig. 1, 3, 7 and 11).

The above results indicate that succinate is not formed in noteworthy amounts from fumarate, malate, citrate, acetate or tartrate during shaking of the yeast suspensions in air or oxygen-free nitrogen previous to the addition of glucose.

The inhibition of alcoholic fermentation thus brought about under anaerobic conditions at pH 4.9 by succinic acid-succinate in yeast cells from young cultures is reversed by molecular oxygen supplemented previous to the addition of sugar (40–44). In the range of 0.005 to 10 or 20 per cent by volume of oxygen in the gas phase the rate of fermentation increases with increasing oxygen concentration, the yeast cells thus behaving contrary to the PASTEUR-MEYERHOF effect as far as the alcoholic fermentation is concerned (44). RUNNSTRÖM et. al (33, 34) as well as BRANDT (5–7) have postulated that succinic acid-succinate is an indifferent buffer not being attacked by the yeast, nor poisoning it. The experimental results described here as well as those reported by WIKÉN and RICHARD (40–44) prove conclusively the incorrectness of this assumption as far as yeast cells from young cultures are concerned. In these cells succinic acid-succinate determines the rate of alcoholic fermentation by interacting with molecular oxygen and one or more components of the zymase system. At moderate succinic acid-succinate concentrations the fermentation inhibition produced by the buffer is counteracted by increasing the oxygen tension in the gas phase. In order to get the same rate of fermentation in a more dilute as in a more concentrated solution of the buffer, the oxygen tension has to be decreased in the former system or increased in the latter one, the succinic acid-succinate thus acting as a regulator of the anaerobic, microaerophilic and aerobic character of the alcoholic fermentation. This physiological property of yeast cells of the generations developing during quite early phases of growth is a constituent part of the orderly modification which the yeast population undergoes with age of the culture irrespective of the changes in the chemical composition of the medium taking place during growth of the yeast (42, 43). Further, this property is not related to any substances exchangeable between yeast cells of different generations in the same way as growth-factors between auxo-heterotrophic organisms in mixed cultures, the yeast cells from young cultures showing a typical succinic acid-succinate effect also in mixtures with insensitive cells from old cultures of the same yeast (43; cf. Fig. 12).

The zymase system which is "masked" in resting young yeast material in presence of succinic acid-succinate by flushing with oxygen-free nitrogen, argon, hydrogen or carbon dioxide is "unmasked" by supplementing the system with molecular oxygen previous to the addition of sugar (as to the phenomenon of "masked enzymes" see Kluyver (12) p. 117). This interference of succinic acid-succinate, molecular oxygen and the zymase of resting cells from young yeast cultures will be discussed in a later paper in relation to the concepts of ,,adaptive'', ,,constitutive'' and ,,induced enzymes'' introduced by Karström (10, 11) and Monod (20, 21), respectively.

The experimental results described in the present paper confirm those reported earlier (40–43) as regards the behaviour of cells harvested from old cultures. These cells are insensitive or show at most only a slight sensitivity under the conditions applied. In the latter case the rate of fermentation is stimulated at low and inhibited at high concentrations of succinic acid-succinate as compared to the rate observed in distilled water (Fig. 12).

The succinic acid-succinate effect upon the alcoholic fermentation in suspensions of resting yeast cells from young cultures appears not only in buffer solutions prepared with sodium hydroxide (40–44) but also in those made with potassium hydroxide (Fig. 2–6). This proves conclusively that the effect is not related to the ratio of potassium to sodium ions. Further, the effect cannot be due to an interference with the potassium ions playing an essential role for the reversibility of the dephosphorylation of phosphopyruvic acid according to Lardy and Ziegler (14).

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# EFFECT OF STREPTOMYCIN ON ORGANISMS SURVIVING THE ACTION OF PENICILLIN

by

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**with technical assistance of N. PEUCHEN-DROST and E. BÜCHLI**

(Received July 12, 1956).

In our previous publications (1955, 1956) we described the survival of a fraction of the numbers of *Staphylococcus aureus*, *Streptococcus faecalis* and *Streptococcus viridans*, after a 7 hours' contact with penicillin. The ability to survive was interpreted by us as the result of structural changes in the bacteria, caused by the action of the antibiotic. The extent of these changes appeared to be proportional to the concentration of the penicillin and to the period of contact. The present publication aims at confirming these assumed structural changes by investigating the effect of streptomycin on these organisms.

Materials and methods have been described in the previous publications. In preparing the Levinthal-media for *Haemophilus influenzae*, the heated blood was not filtered.

The number of bacteria in the inoculum was chosen in such a way that the number of survivors could be easily compared with $10^{-3}$ to $10^{-5}$ dilutions of a standard culture. Inocula of other density have been used in parallel series, the results of which were in accordance with the data recorded in the tables.

**Experiment 1.** The sensitivity to streptomycin of these surviving organisms was compared with that of untreated bacteria. *Haemophilus influenzae* was used as a test organism. Two discs of blotting-paper, each containing 25 $\mu$g of streptomycin were placed on a Levinthal-agar medium in order to obtain two areas with gradually decreasing concentrations of streptomycin towards the periphery. The medium was kept at room temperature overnight. Next morning the discs were removed and the medium was inoculated with one drop of a 18 hours' Levinthal broth culture.

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[1] Working with a grant of the foundation „De Drie Lichten".

Subsequently one disc containing 100 U of penicillin was placed on the exact spot from which a streptomycin disc had been removed, and a second disc with 100 U of penicillin was put on a third spot, such that the three spots formed a triangle. Thus three areas were obtained: one containing streptomycin, the second penicillin, and the third both antibiotics. After another 8 hours these discs were removed and a replica was made on a Levinthal-medium free from antibiotics, which was then incubated during 18 hours. In the replicas the three areas could be clearly distinguished. Although approximately 999 per 1000 organisms had been killed off in the penicillin area, the number of survivors was still important. In both the streptomycin and the penicillin + streptomycin area survivors were rare, but the size of the zones was different. The radius of the zone cleared by both antibiotics was distinctly larger than the radius of the streptomycin zone, but equal to the radius of action of the penicillin.

The lethal concentration of streptomycin seems to be lower for penicillin survivors than for untreated *Haemophilus influenzae.* In order to verify this conclusion we have carried out a slightly altered experiment.

**Experiment 2.** A drop of a 18 hours' culture of *Haemophilus influenzae* (sensitive to $\frac{3}{4}$ U/ml of penicillin and 2 $\mu$g/ml of streptomycin) was inoculated on media containing either 1 U of penicillin, 1 $\mu$g of streptomycin or a combination of these quantities. After 7 hours incubation a replica was made on a plain Levinthal-medium (Table 1). The frequency of the colonies on the replica-plates was compared with the number of colonies grown from a known dilution of a standard culture. The concentration of streptomycin – ineffective when used separately – appeared to reduce the number of survivors of the population from 1 in 300 to 1 in 30.000.

**Experiment 3.** The same experiment was carried out more extensively with *Staphylococcus aureus* strain 1375, which we have used repeatedly in our previous experiments. We intended to study the effect of sublethal concentrations of streptomycin when combined with different concentrations of penicillin (Table 2). The number of survivors of series a) using media containing peni-

TABLE 1.

*Haemophilus influenzae* strain 5. The frequency of the colonies grown on the replica plates was estimated by comparison with the number of colonies grown from 0.5 ml of tenfold dilutions of a standard culture.

| Concentrations of antibiotic in primary medium | | Frequency of colonies on replica plate |
|---|---|---|
| streptomycin | 1 μg | —1 |
| penicillin | 1 U | —2 . —3 |
| penicillin + streptomycin | 1 U + 1 μg | —4 . —5 |
| no antibiotic | | —1 |

—1 = ca. $10^{-1}$; —2 . —3 = ca. 1/300; —4 . —5 = ca. 1/30000.

TABLE 2.

*Staphylococcus aureus* 1375. Effect of various concentrations of penicillin separately or in combination with low concentrations of streptomycin. The ratio a/ab stands for the degree of reduction of survivors induced by streptomycin. Inoculum .01 dilution of a 24 hours' culture.

| Concentration in primary medium of penicillin and streptomycin | Frequency of colonies on replica plates made after hours | | | |
|---|---|---|---|---|
| | 4 | 7 | 9 | 13½ |
| a′ | —3 . —4 | —4 | —4 . —5 | —6 . —7 |
| a′b′ | —3 . —4 | —4 | —4 . —5 | —6 . —7 |
| a′b″ | —3 . —4 | —4 . —5 | —4 . —5 | —6 |
| a′/a′b′<br>a′/a′b″ | 1 | 1 | 1 | 1 |
| a″ | —3 | —3 . —4 | —4 | —5 |
| a″b′ | —3 . —4 | —3 . —4 | —4 . —5 | —5 |
| a″b″ | —3 . —4 | —4 . —5 | —5 | —6 . —7 |
| a″/a″b′ | < 10 | < 10 | < 10 | 1 |
| a″/a″b″ | < 10 | 10 | 10 | > 10 |
| a‴ | —3 . —4 | —3 . —4 | —4 | —5 |
| a‴b′ | —3 . —4 | —5 | —5 | —6 . —7 |
| a‴b″ | —4 | —4 . —5 | —5 | —6 . —7 |
| a‴/a‴b′ | 1 | > 10 | 10 | > 10 |
| a‴/a‴b″ | < 10 | 10 | 10 | > 10 |
| b′ | —2 | —2 | —2 . —3 | —2 |
| b″ | —3 | —3 . —4 | —4 | —4 |

a′ = .08 U/ml penicillin
a″ = 1 U/ml penicillin
a‴ = 50 U/ml penicillin

b′ = 1 μg/ml streptomycin
b″ = 2 μg/ml streptomycin

cillin 0.08 U, 1 U or 50 U was compared with series b′) or b″) containing a same concentration of penicillin in addition to 1 μg or 2 μg streptomycin. After a 4 hours′ incubation the replicas of the a) and b) series did not differ markedly nor was the effect of 1 or 2 μg of streptomycin on organisms surviving the influence of .08 U penicillin noticeable after a prolonged period of contact. In the 1 U/ml media of the penicillin series only the adding of 2 μg of streptomycin effected a significant reduction (10 ×); in the 50 U series 90% of the survivors appeared to be killed by 1 μg of streptomycin. When used separately the highest concentration of streptomycin (2 μg/ml) was significantly less effective than when combined with medium or high concentrations of penicillin. Thus we may conclude that for both *Staphylococcus aureus* and *Haemophilus influenzae*, the sensitivity to streptomycin is increased by the action of penicillin; for *Staphylococcus aureus* the more so, if high concentrations of penicillin are used.

**Experiment 4.** In the following experiment an attempt was made to confirm these results by exposing the staphylococci to penicillin and subsequently to streptomycin; .05 ml of a 24 hours′ culture was inoculated on media containing .08 U, 1 U and 50 U of penicillin. After various periods of incubation replicas were made on intermediate media containing 1 or 2 μg of streptomycin. After another 1, 3 and 7 hours, replicas were made from these intermediate media on plain agar (tertiary) media. In the control series replicas were made from the penicillin (primary) medium to an intermediate plain agar plate, the latter immediately followed by a replica to a (tertiary) plain agar medium. In both series the organisms were spread over the tertiary media in order to compare the actual number of organisms transferred (Table 3).

The number of survivors killed on the intermediate streptomycin medium can be estimated by comparing the frequency of colonies on the tertiary media of both series. A tenfold reduction was not observed in the experiment recorded in table 3 (nor in a parallel series when 2 μg of streptomycin was used). On the contrary, as compared with the control series, a slight increase (i.e. growth) of the number of survivors could be seen after prolonged incubation on the streptomycin media, and the more so, when the organisms originated from media containing critical concentrations of penicillin (.08 U/ml).

TABLE 3.

*Staphylococcus aureus*, strain 1375, exposed to penicillin and streptomycin consecutively and vice versa.

| Pre-incubation on primary medium (in hours) | Concentration in primary medium penicillin U/ml | Frequency of survivors on tertiary medium | | | |
|---|---|---|---|---|---|
| | | control series (no streptomycin) | incubation on intermediate streptomycin in hours | | |
| | | | 1 | 3 | 7 |
| 1 | .08 | —1 . —2 | —1 | —1 | —1 |
| | 1 | —1 . —2 | —1 . —2 | —1 | —1 |
| | 50 | —1 . —2 | —1 . —2 | —1 . —2 | —1 |
| 3 | .08 | —2 | —2 . —3 | —1 | —1 |
| | 1 | —2 . —3 | —3 | —3 | —1 . —2 |
| | 50 | —3 | —3 | —3 | —2 . —3 |
| 7 | .08 | —4 . —5 | —4 | —3 . —4 | —2 . —3 |
| | 1 | —4 | —4 . —5 | —3 . —4 | —2 . —3 |
| | 50 | —3 . —4 | —3 . —4 | —4 | —3 |
| | streptomycin μg/ml | 6 hours' incubation on intermediate penicillin medium containing 50 U/ml | | | |
| 6 | 1 | —2 . —3 | | | |
| | 0 | —2 . —3 | | | |

**Experiment 5.** In a former series of experiments (1956) we observed for both *Staphylococcus aureus* and *E. coli* that penicillin treated organisms will develop colonies at a slower rate than untreated bacteria. We moreover mentioned the conformity of the morphologic changes caused by penicillin in different species of bacteria, as reported by various authors.

So we investigated whether the combination of the high concentrations of penicillin and sublethal concentrations of streptomycin, observed to be highly effective against *Staphylococcus aureus* and *Haemophilus influenzae*, is equally effective against other species. As recorded in table 4, this combination brought about a marked reduction in the number of survivors of both *E. coli* and *Proteus mirabilis* populations.

With a strain of *Vibrio El Tor* we have carried out experiment 1 as described for *Haemophilus influenzae*, with similar results.

**Experiment 6.** A penicillin-streptomycin combination was tested against a strain of *Haemophilus influenzae* and a α-haemolytic

TABLE 4.

Frequency of colonies of *E. coli* K 12 and *Proteus mirabilis* 494, grown from organisms which survived the antibiotic action.

| Strain | Concentration in primary medium | | Frequency of colonies on replica | | | |
|---|---|---|---|---|---|---|
| | | | incubation in hours on primary medium | | | |
| | penicillin U/ml | streptomycin µg/ml | 3 | 6 | 9 | 14 |
| *E. coli* K 12 | — | 1 | –1 | –1 | –1 | –1 |
| | 500 | — | –4 | –4.–5 | –5 | –6 |
| | 500 | 1 | –4 | –5 | –6 | — |
| *Proteus mirabilis* 494 | — | 1 | 1 | 1 | 1 | 1 |
| | 500 | — | –3 | –4 | –5 | –5 |
| | 500 | 1 | –3 | –5 | –6 | — |

TABLE 5.

Diameter of the clear zone and frequency of colonies of α-haemolytic *Streptococcus* 1191, *Haemophilus influenzae* 1475 and *Staphylococcus aureus* ,,Oxford'' (substrain made resistant in vitro to 100 µg of streptomycin).

| Strain | Antibiotic in primary medium | Replica 7 hours | | Replica 24 hours | |
|---|---|---|---|---|---|
| | | diameter of clear zone in mm | frequency of colonies | diameter of clear zone in mm | frequency of colonies |
| *Streptococcus* 1191 | penicillin | 25 | —4 | 30 | 10 k |
| | penicillin + streptomycin | 26 | —4 | 30 | 10 k |
| *Haemophilus* 1475 | penicillin | 30 | —4 | 38 | 2 k |
| | penicillin + streptomycin | 30 | —4 | 39 | 2 k |
| *Staphylococcus* ,,Oxford'' substrain rSm | penicillin | 34 | 25 k | | |
| | penicillin + streptomycin | 34 | 32 k | | |

Small numbers of colonies counted (k).

streptococcus, both resistant to 100 µg of streptomycin (but sensitive to penicillin) and an "Oxford" strain of *Staphylococcus aureus* which was made resistant in vitro to this concentration of streptomycin. The frequency of the colonies, their distribution and the radii were the same for the replicas of penicillin zones on the one hand and the penicillin plus streptomycin zones on the other (Table 5). The organisms that had survived the action of penicillin were resistant to streptomycin in the same measure as the original strains, so the effect of streptomycin was nil.

**Experiment 7.** Two penicillinase producing strains of *Staphylococcus* were studied next. One of these (4886, resistant to 5 U, sensitive to 10 U/ml of penicillin) was considered a weak penicillinase-producer. Of the other strain (A 67) 1 in 100 organisms (inoculum 1/100 ml of a 24 hours' culture) developed into colonies on a 100 U/ml penicillin medium. Both strains produced a sharp outline of the inhibition zone around the penicillin disc (considered by us as characteristic for penicillinase-producers (1953)). With both strains there was a close (inverse) relationship between the amount of the inoculum and the inhibitory concentration of penicillin.

TABLE 6.

Effect of penicillin and streptomycin on two penicillinase producing Staphylococci.

| Strain of *Staphylococcus* | Contact on primary medium in hours | Concentration of antibiotic in primary medium | | | | | |
|---|---|---|---|---|---|---|---|
| | | penicillin U | — | 50 | 50 | 500 | 500 |
| | | streptomycin µg | 1 | — | 1 | — | 1 |
| A 67 inoculum $10^{-2}$ sensitivity > 100 U | 3 | | 1 | –4 | –4 | –4 | –5 |
| | 6 | | 1 | –4.–5 | –4.–5 | –4.–5 | –6 |
| | 9 | | 1 | –5 | –5 | –6 | –6 |
| | 14 | | 1 | — | –6 | — | — |
| 4886 inoculum $10^{-1}$ sensitivity 5–10 U | 3 | | 1 | –3 | –3.–4 | –3.–4 | –4.–5 |
| | 6 | | 1 | –4 | –4 | –4 | –5 |
| | 9 | | 1 | –4 | –4 | –5.–6 | –6 |
| | 14 | | 1 | –4.–5 | –4.–5 | –5 | — |
| | | | Frequency of colonies | | | | |

In order to decrease the influence of the penicillinase production the inoculum was reduced to a 1/100 dilution (of a 24 hours' culture) for strain A 67 and a 1/10 dilution for strain 4886. Moreover we used very high concentrations of penicillin. Under these conditions the combination of penicillin + streptomycin proved to be much more effective than its separate components (Table 6).

**Experiment 8.** In this experiment the influence of chloramphenicol upon the action of penicillin and upon the combination penicillin-streptomycin was studied. In order to obtain an approximately equal number of survivors of both penicillin + chloramphenicol and penicillin media, the density of the inoculum of the latter was a hundredfold that of the former. By adding 1 μg of streptomycin to the medium, the reduction of survivors was 1 in 10 in either case. This concentration of streptomycin had no effect upon the number of survivors of chloramphenicol alone (Table 7).

TABLE 7.

*Staphylococcus aureus* 1375. Penicillin + chloramphenicol. Effect of streptomycin on surviving organisms.

| Inoculum | Concentration in primary medium | | | Period of incubation on primary medium (in hours) | | | | Reduction by streptomycin |
|---|---|---|---|---|---|---|---|---|
| | penicillin U/ml | streptomycin μg/ml | chloramphenicol μg/ml | 6 | 9 | 14 | 24 | |
| 1/100 | — | 1 | — | –2 | –2 | –2 | –2 | |
| 1/100 | — | — | 10 | –3 | –3.–4 | –4.–5 | –6 | 1 |
| 1/100 | — | 1 | 10 | –3 | –3 | –5 | –6 | |
| 1/1 | 50 | — | — | –2.–3 | –3 | –3.–4 | –5 | 1/10 |
| 1/1 | 50 | 1 | — | –3.–4 | –3.–4 | –4.–5 | –6 | |
| 1/100 | 50 | — | 10 | –3.–4 | –3.–4 | –4 | –5 | 1/10 |
| 1/100 | 50 | 1 | 10 | –4 | –4.–5 | –5 | –5.–6 | |

## DISCUSSION.

The investigation was begun in 1953 in order to obtain a better understanding of the mode of action of the penicillin-streptomycin combination. The number of bacteria surviving a several hours' contact of this combination had appeared considerably lower than after contact with penicillin alone. In the discussion of our first series of experiments (1955) we suggested that the survivors of high concentrations of penicillin were killed by low concentrations of streptomycin. In the following publication (1956) further details were given as to the character of the organisms that had survived the action of penicillin. We interpreted the phenomena observed, by assuming the existence of reversible structural changes, the extent of which was dependent on the concentration of penicillin.

In our present experiments organisms surviving high penicillin concentrations were sensitive to 1 μg of streptomycin; a 2 μg concentration was needed to kill off 90% of the organisms surviving a medium penicillin concentration. The organisms surviving the action of critical concentrations (.08 U/ml) of penicillin were not found to differ from untreated bacteria as to their streptomycin sensitivity. So the lethal concentration of streptomycin was related to the concentration of penicillin used. The present results obtained with *Staphylococcus aureus, Haemophilus influenzae, Proteus mirabilis, Escherichia coli, Vibrio El Tor,* thus confirmed our former assumption as to the structural changes effected by penicillin. We surmised moreover the connection of these structural changes with the "L" cycle. TULASNE and MINCK (1952) have stated that "L" forms of *Proteus* are much more sensitive to streptomycin than the bacterial form.

The increase in effect of sublethal concentrations of streptomycin on penicillin-treated staphylococci was only observed when both acted simultaneously. Even after a 7 hours' contact with high concentrations of penicillin, the surviving organisms were not significantly reduced in numbers by subsequent contact with sublethal concentrations of streptomycin. After a certain lag-time, the duration of which was shortest for low concentrations of penicillin in the primary plates, even a slight increase could be observed. These results are in agreement with those published by MILES, COLEMAN, GUNNISON and JAWETZ (1951) who used a different technique.

Strains resistant to high concentrations of streptomycin do not become sensitive to streptomycin during exposure to penicillin. In two penicillinase producing strains, however, a phase sensitive to sublethal concentrations of streptomycin could be induced by using very high concentrations of penicillin. The sensitivity to penicillin of a single penicillinase producing *Staphylococcus* is comparable to a sensitive one. Of a population of penicillinase producers the inhibitory concentration of penicillin is dependent on the amount of penicillinase formed.

The action of a chloramphenicol and penicillin combination is less effective and slower than of penicillin alone. In both cases 90% of the surviving organisms are killed by sublethal concentrations of streptomycin. This would suggest that in a part of the population a reversible change is induced by the penicillin after prolonged incubation in spite of the inhibitory action of chloramphenicol.

It is noteworthy that two chronic diseases: subacute bacterial endocarditis and bronchitis caused by *Haemophilus influenzae* (MULDER, GOSLINGS, VAN DER PLAS, LOPES CARDOZO (1952)) are successfully treated with the combination of high concentrations of penicillin and low concentrations of streptomycin.

## Summary.

The sensitivity to streptomycin of several species of bacteria (*Haemophilus influenzae, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Vibrio El Tor*) appears to be increased by the simultaneous action of high concentrations of penicillin. With Staphylococci this increase is found to be proportional to the concentration of penicillin.

In vitro, the penicillin-streptomycin combination is also markedly effective towards penicillinase producing staphylococci. Towards streptomycin resistant organisms, however, this combination does not seem to be more effective than penicillin alone.

Though the number of survivors of the penicillin-chloramphenicol combination is a multiple of the number surviving penicillin alone, the reduction brought about by adding sublethal concentrations of streptomycin is 90% in either case.

The results of these experiments confirm our assumption of reversible structural changes caused by the action of penicillin, the extent of these changes being dependent on the concentration of this antibiotic.

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# PATHOGENICITY OF *CORYNEBACTERIUM BELFANTI* FOR THE CHICK EMBRYO

by

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The use of a term *Corynebacterium belfanti* was suggested by one of us (Bezjak 1954, 1955 and 1956) in order to denote atoxic strains of *C.diphtheriae* type *mitis* found in nasal discharge from patients with atrophic rhinitis. It was noticed that *C.belfanti* could be differentiated from atoxic mitis strains by the negative nitrate reduction test as well as by the slower glucose fermentation (Parsons-Frobisher test).

*C.belfanti* strains are deprived of any diphtheria toxin though some strains cause local reactions when injected intracutaneously into rabbits (Bezjak 1956). In mice inoculated intracerebrally with *C.belfanti* the presence of an endotoxin similar to that observed by Frobisher and Parsons (1940) in *C. diphtheriae* type *mitis* strains was detected too (Bezjak 1956).

Some authors investigated the pathogenicity of *C. diphtheriae* for chick embryos and came to different conclusions. Thus, T'ung (1945) observed that atoxic diphtheria bacteria produced pseudomembrane formation on the chorioallantoic membrane. Knothe (1952) in contrast, drew the conclusion that there is no difference between the results in guinea-pigs and chick embryos; only toxic *C. diphtheriae* strains caused edematous thickening and greyish-white opacities of the chorioallantoic membrane.

To obtain a more precise information of the pathogenic potentialities of *C.belfanti*, the investigation of its pathogenicity for the developing chick embryo was undertaken.

## Materials and methods.

Strains. Thirty strains of *C.belfanti* isolated from nasal discharge of ozaena patients were used. All the strains proved atoxic when tested in animals or by the Ouchterlohny's method, and differed from the *mitis* type of *C.diphtheriae* by the negative nitrate reduction test.

Inoculum. Prior to the inoculation into eggs the strains have been grown on Löffler slants for 24 to 48 hours.

Bacterial suspensions were prepared by emulsifying one loopful (3 mm in diameter) of bacterial growth in 0.5 ml saline. One to two drops of this suspension were inoculated into the egg by the aid of a capillary pipette of the medium size.

Method. Only well developed 10 days old embryonated Leghorn eggs were used. All the eggs were inoculated onto the chorioallantoic membrane. During incubation at 37°C. they were daily inspected and immediately after the death of the embryo opened and examined. The examination consisted of staining smears from pseudomembranes or nodules seen on the membrane surface, inoculation of blood-agar plates with material taken from membranes, close inspection and description of the membrane changes and of a histologic study of membranes preserved in 10 per cent solution of formaldehyde.

## RESULTS.

### Bacteriology.

Pathogenic action of 30 strains of *C. belfanti* on the developing chick embryo by inoculation of chorioallantoic membrane was investigated. With each strain one inoculation was made, while with seven strains of *C. belfanti* recovered from the chorioallantoic membrane during the embryo's life, the experiment was repeated. Thus, in total 37 inoculations were carried out.

All the inoculated eggs have been daily observed and after the embryos' death they have been opened and examined. In 12 cases chorioallantoic membranes have been taken out at various periods after the inoculation while the embryos were still alive. This was done for the purpose of studying membrane changes during the embryos' life. In one instance an embryo survived the infection and the chick was hatched on the 26th day of incubation.

The moment of the embryo's death as result of the pathogenic action of *C.belfanti* strains inoculated, varied somewhat. Eleven strains of *C. belfanti* killed chick embryos after two days, six after

3 days and three strains 4 days following inoculation; 4 embryos died after 6, 7, 8 and 12 days respectively.

In 12 instances inoculated eggs were opened after two (1 egg), three (2 eggs), five (5), seven (1), eight (1), twelve (1) or fourteen days (1) during the embryo's life.

In smears taken from the pathologically changed chorioallantoic membranes and stained according to Gram and Lubinsky many morphologically typical rods with metachromatic granules were found. Cultivation of this material yielded in the majority of cases pure cultures of *C.belfanti*. In two instances (case No 15 and 21) inoculated blood-agar plates remained sterile, though the chorioallantoic membranes showed minor pathologic changes. The same was observed in two cases (No 9 and 13) where the eggs had been opened before the embryos had died; strain No 9 was apathogenic and produced no pathologic lesions, while strain No 13 caused only minor membrane lesions.

In seven instances (cases No 15, 16, 17, 18, 20, 21 and 25), where inoculated eggs have been opened before the death of the embryo, fresh embryonated eggs were infected with the same strains of *C. belfanti* recovered from the first passage. In the second passage the enhanced pathogenicity was noted as seen by a shortening of the time in which the embryos died, but more pronounced lesions were observed in only two of the six membranes (No 16 and 25). One embryo (case No 18) survived the infection and on its 26th day the chick showing digital malformations was hatched; five days later it succombed to an intercurrent infection.

Even from this small number of experiments it is evident that a same strain of *C. belfanti* may cause different macroscopic chorioallantoic membrane lesions. For comparison all the data are taken up in Table 1.

Finally, the effect of various numbers of bacterial cells on the virulence for the developing chick embryos was studied. For this purpose *C. belfanti* strain No 3 was used. With tenfold dilutions of the bacterial suspension, containing 200, 20 and 2 million and 200.000 cells, made from the 48 hour old blood-agar culture, groups of five eggs were inoculated onto the chorioallantoic membrane.

A total of 12 embryos died during this experiment: five succombed to *C. belfanti* infection, one following an incidental injury at the time of inoculation, the remaining six died during the hatching for an unknown reason, some of them probably damaged by the

TABLE 1.

Results in the first and second passage through embryo of seven *C. belfanti* strains.

| Case No. | First passage: eggs opened alive after | Second passage: embryo died after | Chorioallantoic membrane lesions | |
|---|---|---|---|---|
| | | | First passage | Second passage |
| 15 | 5 days | 3 days | hemorrhages | few nodules |
| 16 | 8 days | 3 days | none | many nodules and pseudomembrane |
| 17 | 7 days | 2 days | nodules and pseudomembrane | pseudomembrane |
| 18 | 3 days | * | few nodules | * |
| 20 | 5 days | 2 days | pseudomembrane and hemorrhages | many nodules and hemorrhages |
| 21 | 5 days | 4 days | few nodules and hemorrhages | few nodules |
| 25 | 5 days | 4 days | few nodules | many nodules, pseudomembrane and hemorrhages |

* In the second passage a chick was hatched.

infection with *C. belfanti.* Four of the five embryos infected with 200 million cells died in 1 to 2 days, while one out of five embryos inoculated with 20 million cells had succombed after one day. The results are seen in Table 2.

TABLE 2.

Results of the number of *C. belfanti* cells (strain No. 3) on the course of infection in embryonated eggs.

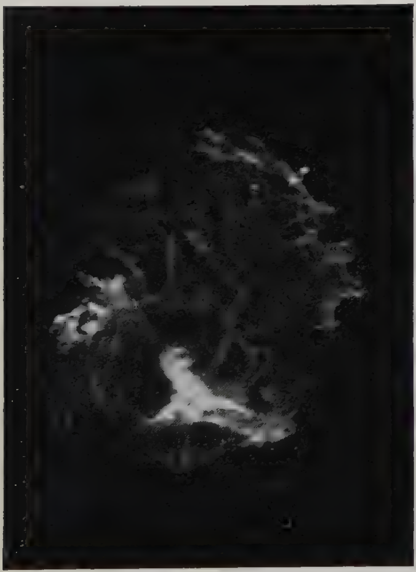
| Embryo No. | Chorioallantoic membranes inoculated with | | | |
|---|---|---|---|---|
| | 200 million cells | 20 million cells | 2 million cells | 200.000 cells |
| 1 | died in one day | died in one day | died during hatching | died in one day* |
| 2 | died in one day | died during hatching | died during hatching | a chick was hatched |
| 3 | died in one day | a chick was hatched | died during hatching | a chick was hatched |
| 4 | died in two days | a chick was hatched | died during hatching | a chick was hatched |
| 5 | a chick was hatched | a chick was hatched | died during hatching | a chick was hatched |

* Died from an incidental injury.

Among the eight chickens hatched during this experiment, five had digital malformations and contractures. Three of them died after 4, 8 and 11 days respectively, but no bacteria were found in blood or internal organs at the postmortem examination. Therefore, the cause of these deaths could not be determined.

Gross pathology.

The gross pathology of chorioallantoic membranes was not of the uniform type and the changes could be classified into several groups. The lesions were quite often of the pseudomembrane type (Fig. 1) which varied in number (one to 4 per membrane) and in size (from few to even 150 mm²). Small whitish nodules which also showed variations in size up to 2 millimeters and which sometimes were more like whitish spots, occurred quite regularly. These nodules were usually associated with the presence of a pseudomembrane (Fig. 2). Pseudomembranes could easily be removed with a platinum loop, while nodules were more attached to the chorioallantoic membrane. In one instance (case No 25) membranes were much thickened and of a gelatinous appearance.



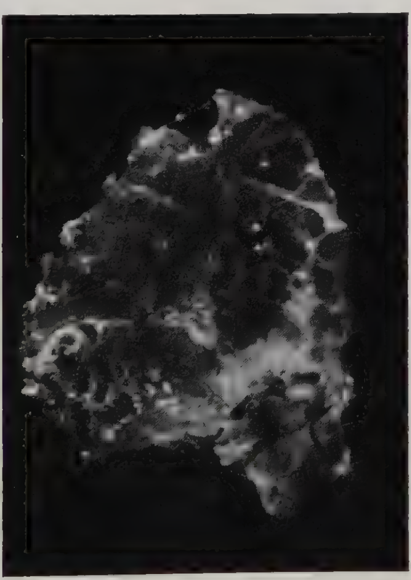


Fig. 1. Pseudomembrane type of chorioallantoic membrane lesions (*C. belfanti* strain No. 30).

Fig. 2. Pseudomembrane and many nodular lesions of the chorioallantoic membrane (*C. belfanti* strain No. 8).

Thirteen chorioallantoic membranes infected with 12 strains of *C. belfanti* showed hemorrhages of the more diffuse type (in 9 cases) or in the petechial form (4 cases). Hemorrhages of the first type were not of a traumatic origin as judged by the color and the character. Hemorrhagic lesions were usually associated with lethal infections, while in three cases they have been detected in membranes of the living embryos. One membrane (case No 15) showed macroscopically only hemorrhagic lesions of both types.

All chorioallantoic membranes of the embryos having succombed during the experiment showed pathologic lesions, while three membranes of living embryos were without any macroscopic changes (cases No 9, 16 and 19). Membrane lesions were classified according to the degree of pathologic changes as follows:

a) many nodules accompanied by pseudomembrane;
b) pseudomembranes only;
c) many nodules without pseudomembranes;
d) few nodules only;
e) lesions associated with hemorrhages;
f) normal chorioallantoic membranes without any lesions.

All the results of macroscopic and histologic examination are presented in Table 3.

The most frequent type of chorioallantoic lesions in embryos which had died during the experiment was that op pseudomembranes associated with many nodular lesions, and observed in 11 cases. Exclusive pseudomembrane formation was found in four instances, many nodules were seen in three, while occasional nodules were detected in six cases.

The distribution of lesions according to the type and degree was different in chorioallantoic membranes taken out during the life of embryos. In this group in five cases scattered nodular lesions were found. In three cases no visible lesions could be detected, not even with the aid of a lens, while in one only hemorrhages were present. The results of membrane gross pathology from both the embryos dead or alive are shown in Table 3 and 4.

## Histology.

Eighteen chorioallantoic membranes have been studied histologically. All the membranes have been fixed in formaldehyde, embedded in paraffin and the sections stained by hemalum-eosin and by the Weigert-Gram's modification respectively.

## TABLE 3.

Results of gross pathology and histology in chorioallantoic membranes inoculated with 30 strains of *C. belfanti*.

| Case No. | Strain No. | Embryo | | Gross findings 3) | Histological finding | Remarks |
|---|---|---|---|---|---|---|
| | | died after | opened alive after | | | |
| 1 | 26532 | 12 days | | C | ... | ... |
| 2 | 26745 | - | 3 days | D | Granulation tissue, vasodilatation | ... |
| 3 | 26746 | 9 days | | A | ... | ... |
| 4 | 27007 | 2 days | | A | Edema, infiltration, necrosis | ... |
| 5 | 27126 | 3 days | | A | Infiltration, necrosis | ... |
| 6 | 27206 | 3 days | | A & E | Infiltration, necrosis, vasodilatation | ... |
| 7 | 27248 | 6 days | | D | Infiltration, necrosis | ... |
| 8 | 27507 | 3 days | | A | Ulceration, infiltration, necrosis | ... |
| 9 | 27794 | | 14 days | F | ... | No growth in culture |
| 10 | 27795 | 3 days | | A | Edema, necrosis, reactive inflammation | ... |
| 11 | 3108 | 2 days | | A & E | ... | ... |
| 12 | 17125 | | 5 days | C | Infiltration | ... |
| 13 | 17249 | | 12 days | D | ... | ... |
| 14 | 19467 | 4 days | | B & E | Edema, necrosis, reactive inflammation | ... |
| 15a | 20067 | | 5 days | F / E 2) | Edema, reactive inflammation | ... |
| 15b 1) | 20067 | 3 days | | D | ... | No growth in culture |
| 16a | 22157 | | 8 days | F | ... | ... |
| 16b 1) | 22157 | 3 days | | A | ... | ... |
| 17a | 22664 | | 7 days | A | ... | ... |
| 17b 1) | 22664 | 2 days | | B | ... | ... |
| 18a | 22813 | | 3 days | D | Edema, infiltration, necrosis, granulation tissue | ... |
| 18b 1) | 22813 | ... | | ... | ... | A chick was hatched |
| 19 | 22818 | | 2 days | F | Nil abnormal | ... |
| 20a | 23439 | | 5 days | B & E | Edema, infiltration, necrosis, reactive inflammation | ... |
| 20b 1) | 23439 | 2 days | | C & E | ... | ... |
| 21a | 24929 | | 5 days | D & E petechial | Edema, infiltration, reactive inflammation | ... |
| 21b 1) | 24929 | 4 days | | D | ... | No growth in culture |
| 22 | 25030 | 7 days | | A & E | ... | ... |
| 23 | 25053 | 2 days | | C & E | Small necroses | ... |
| 24 | 25207 | 2 days | | D & E | No lesions; bacterial aggregates | ... |
| 25a | 26507 | | 5 days | D | Edema, vasodilatation, perivascular infiltration | ... |
| 25b 1) | 26507 | 4 days | | A & E | ... | ... |
| 26 | 27965 | 2 days | | A | Infiltration | ... |
| 27 | 49054 | 2 days | | D & E petechial | ... | ... |
| 28 | 51244 | 2 days | | B & E petechial | ... | ... |
| 29 | 4094 | 2 days | | D | ... | ... |
| 30 | 4320 | 2 days | | B | ... | ... |

1) Second passage through embryo. 2) Hemorrhages were the sole lesions.

3) A many nodules and pseudomembrane; B pseudomembrane(s) only; C many nodules; D few nodules: E hemorrhages; F nil abnormal.

TABLE 4.

Chorioallantoic membrane lesions in embryos died from *C. belfanti* infection and in those infected but still living.

| Membrane lesions | Number of embryos | | |
|---|---|---|---|
| | Died | Alive | Totals |
| Many nodules and pseudomembrane(s) | 11 | 1 | 12 |
| Pseudomembrane(s) only | 4 | 1 | 5 |
| Many nodules | 3 | 1 | 4 |
| Few nodules | 6 | 5 | 11 |
| No lesions | — | 5 [1] | 5 |
| Hemorrhagic lesions | 10 | 3 | 13 |

[1] In one case (No. 15) only hemorrhages; in another case (No. 18) in the second passage chorioallantoic membrane was not available because a chick was hatched, while in the first passage few nodules appeared.

In sections of all but three membranes (cases No 12, 19 and 24) histologic changes were observed; in one of these (No 24) *C. belfanti* cells, scattered and in small groups, were seen.

Histologic lesions consisted in the membrane cells' edema (Fig. 3), in raising of the epithelial layer (Fig. 4), in reactive inflammatory thickening of minor or larger portions of membrane with necrosis of epithelial cover and its shaking off (Fig. 5). The changes in the membranes varied in degree and distribution only. All the histologically changed membranes showed the presence of Gram-positive rods with morphologic characteristics of *C. belfanti*. The number of bacteria varied and showed all transitions from scattered rods to small and large accumulations. Bacterial aggregates were usually found under the epithelium, upon the parts where the epithelial cover was destroyed or in the membraneous tissue itself, but rarely on the preserved epithelial layer (Fig. 6).

## Discussion.

The results of this investigation have demonstrated the pathogenic action of *Corynebacterium belfanti*, i.e. atoxic strains of *C. diphtheriae* type *mitis* with negative nitrate reduction test, for the developing chick embryo.The majority of strains caused the death of the embryo in 2 to 4 days following inoculation, though some embryos had succombed after 6 to 12 days. The strains tested after

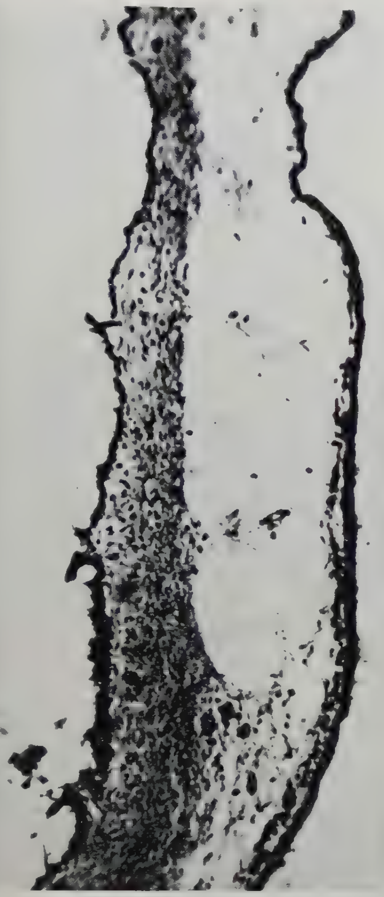


Fig. 3. Oedema of the chorioallantoic membrane. 100 ×.

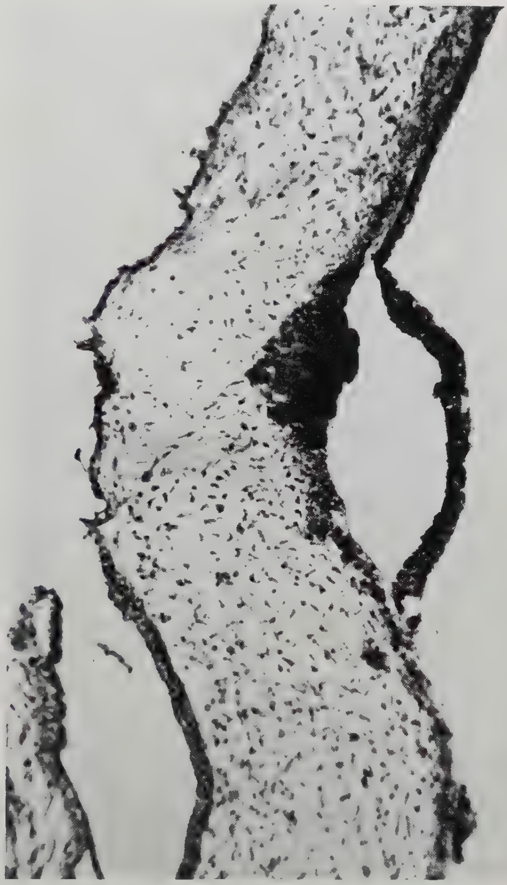


Fig. 4. Cell infiltrate with *C. belfanti* aggregates under the raised epithelial layer. 100 ×.

the first passage through the embryo showed invariably enhanced virulence as judged by the embryos' death at an earlier date.

During the study of the effect of the number of *C. belfanti* cells on the course of infection in embryonated eggs, five out of eight chickens hatched showed digital malformations and contractures. This unusual high percentage of malformations, compared with only few chickens showing such lesions in another much larger group of experiments carried out in this laboratory at the same time but with other organisms, is striking. Therefore, we believe these mal-

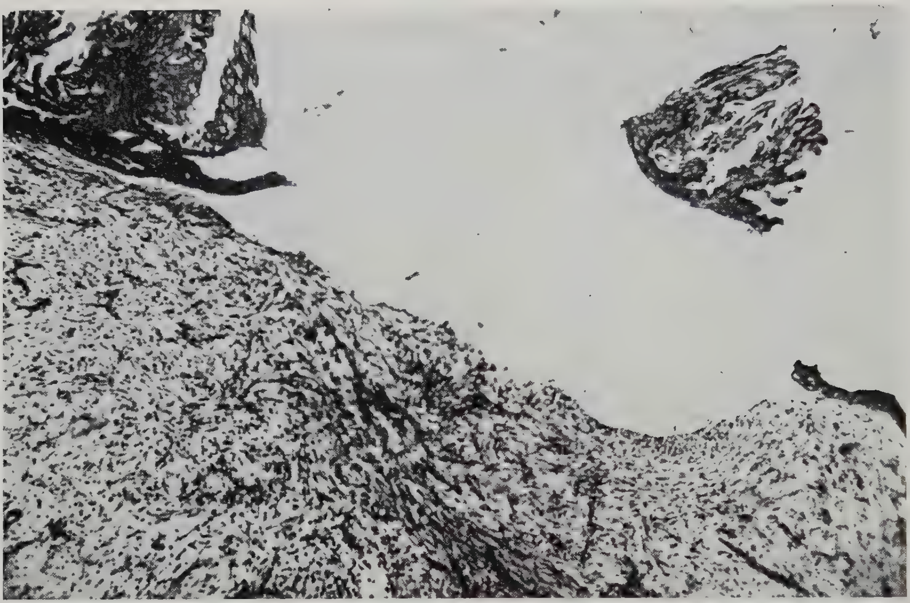


Fig. 5. Deterioration of epithelium and reactive inflammation of the chorioallantoic membrane. 50 ×.

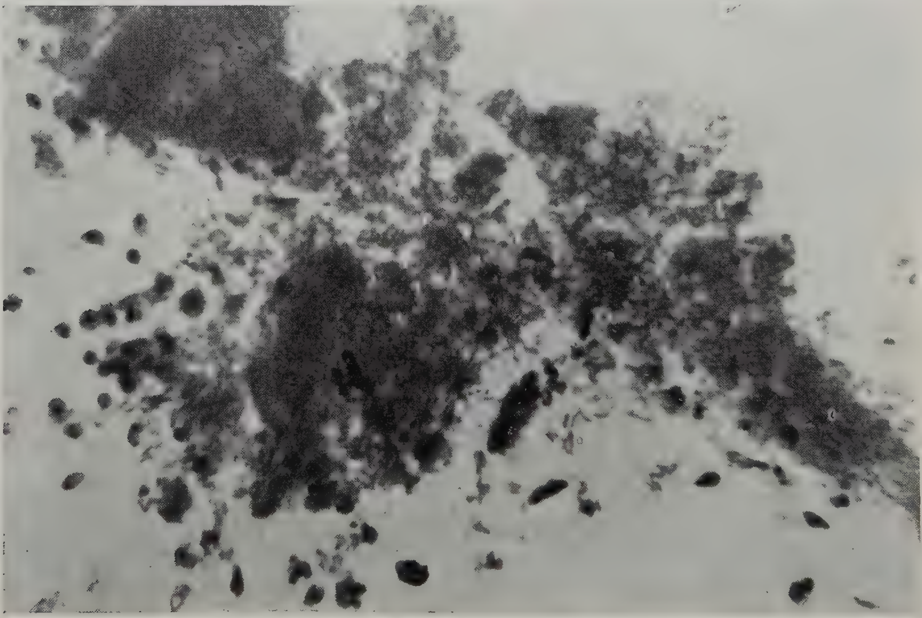


Fig. 6. Bacterial aggregates in epithelial layer. Weigert-Gram. 600 ×.

formations not to result from environmental factors, these having been similar in both groups of eggs, but probably to be due to the neurotropic endotoxin previously demonstrated in *C. belfanti* strains (Bezjak 1956).

This investigation has shown that *C. belfanti* lesions of chorioallantoic membranes consisted macroscopically of pseudomembrane and/or nodules characterized histologically by edema, cell infiltration and necrosis. These pathologic changes were closely related to the lesions observed by other authors experimenting with toxic and/or atoxic strains of *C. diphtheriae.*

Comparing the development and the degree of chorioallantoic membrane changes we may summarize the pathogenesis of these lesions as follows: at the beginning only hyperaemia appears followed by the multiplication of bacteria, at first on the surface of the epithelium and later in the depth of membrane; blood vessels show dilatation and contain many lymphocytes; around the bacterial clumps infiltration with lymphocytes appear as a result of an inflammatory response; the membrane itself reacts with edematous thickening; in the epithelial layer minute areas of necrosis develop and grow in size, and finally the epithelium is shoven off and decays.

## Summary.

Pathogenicity of 30 strains of *Corynebacterium belfanti* by inoculation of chorioallantoic membrane of the developing chick embryo was investigated. The embryos usually died in 2 to 4 days and chorioallantoic membranes showed the presence of nodules and/or pseudomembranes sometimes accompanied by hemorrhages. Histologically the lesions consisted of oedema, cell infiltration and necrosis.

In the second passage through eggs, enhanced virulence of *C. belfanti* was noticed.

During experiments with *C. belfanti* eight chickens were hatched, five of them showing digital malformations and contractures.

All the results are presented in four tables and six figures.

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# VIRUS PNEUMONIA (SNUFFLING DISEASE) IN LABORATORY RATS AND WILD RATS DUE TO AN AGENT PROBABLY RELATED TO GREY LUNG VIRUS OF MICE [1] [2]

by

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Snuffling is a common disease of rats in various laboratories and laboratory animal breeding stations. The aetiology and pathology of infections of the respiratory tract of rats, in which snuffling is a characteristic symptom, do not seem to be uniform. Various microorganisms, i.e. *B. muris*, anaerobic *Streptothrix* species, anaerobic spirillae, haemolytic and non-haemolytic streptococci, diphtheroids, *E. coli*, *Pasteurella bronchiseptica*, *B. actinoides* (5, 6, 7, 10, 16), have been isolated from the lungs, but none of them has been found with a regularity and frequency sufficient for assuming any aetiological relationship with the disease. Since many authors failed to isolate microorganisms from a relatively high percentage of the rat lungs, the microorganisms cultivated might probably be regarded either as secondary invaders, or as incidental contaminants.

KLIENEBERGER and STEABBEN (9) isolated $L_3$ organisms of the group of pleuropneumonia-like organisms (PPLO) from bronchopneumonias and bronchiectasies of rats, however, without evidence of their aetiological significance.

A serially transmissible infectious catarrh has been described by NELSON (11), in which coccobacilliform bodies were observed in leucocytes and epithelial cells. The bodies could be grown in chick embryonic tissue culture and the disease could be produced experimentally with the cultures. Mice proved also susceptible to intranasal instillation of the cultures.

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[1] Aided by a grant from the National Health Research Council T.N.O.

[2] This paper is part of a thesis in preparation by H. VROLIJK, which has not been finished because of the decease of the author.

Different from infectious catarrh is endemic pneumonia of albino rats, which Nelson (12, 13) found to be due to a virus pathogenic for rats and mice, and which might be related to grey lung virus of mice (1), and to a virus pneumonia in wild rats transmissible to mice (17).

In this paper, some of the characteristics of a virus recovered from lungs of rats in various laboratories, and of wild rats, are described. A number of them showed symptoms of snuffling disease, others were apparently healthy. The animals were sacrificed, and in all rats with clinical signs of illness, and in many of the apparently healthy rats, lung lesions were found.

In mild cases, small grey or pinkish areas of consolidation were found in the lungs. On histological examination these areas were characterized by oedematous alveolar walls, which were frequently infiltrated with mononuclear cells, and varying numbers of leucocytes. The alveoli were either compressed, or filled with predominantly mononuclear cells. The bronchioles were surrounded by a lymphocytic infiltration.

In more advanced cases, the lesions were essentially similar, but more extensive. The consolidated areas were extended over larger parts of the lungs; one or more lobes could be involved entirely or almost entirely. Circumscript foci with accumulation of leucocytes were observed in peribronchiolar regions, or even penetrating the walls of bronchioli and bronchi. In these cases, a beginning bronchitis and bronchiolitis was present.

In rats with apparently chronic illness, lung abcesses could be observed already on naked-eye examination.

Usually no microorganisms could be isolated from lungs showing consolidated areas without abcess formation. From chronic cases with abcesses, however, various microorganisms could be cultivated.

## Transmission experiments.

As in Nelson's investigations (12), we had no entirely suitable natural host at hand for experimental transmission, since the natural disease appeared to be widespread in colonies of laboratory rats in the Netherlands. Hence, experimental transmission was attempted in mice.

The rats were sacrificed under anaesthesia, the lungs were removed aseptically, and examined for the presence of microorganisms and the PPLO-group by aerobic and anaerobic cultivation on sheep blood agar, and on KLIENEBERGER's medium consisting of boiled blood, ox-heart infusion peptone, yeast extract and horse serum (8). Pending the result of the bacteriological examination, the lungs were stored in a dry-frozen state at —20°C.

Only lungs from which no microorganisms had been recovered, were used for transmission experiments. The lungs were ground in a tissue grinder, and homogenized with saline to a 20 percent suspension. After centrifuging for 15 minutes at 3000 r.p.m. the supernatant fluid was inoculated by intranasal instillation into mice weighing 8 to 10 grammes, under ether anaesthesia.

When sacrificed 14 days after infection, lung lesions similar to those in the mild natural disease of rats were found in approximately 50 per cent of the mice of the first passage. Subsequent passages were made with mouse lungs that proved bacteriologically sterile. After 3–5 passages, lung lesions were found in 100 per cent of the inoculated mice. Death occurred only exceptionnally. In suckling-mice, however, the experimental pneumonia was usually fatal within 7–14 days.

In order to exclude the possibility of picking up a latent pneumonia-producing agent from the respiratory tract of the mice, each of the first 5 passages was paralelled by an equal number of mice inoculated with a suspension of normal mouse lung, each of the following passages being made with lungs of the preceding passage. Lung lesions were found in none of the control-mice.

The conclusion that the pneumonia in rats is most likely due to a virus, which is also pathogenic for mice, seems to be justified.

In the aggregate 78 rats, among which 2 wild rats, have been examined for the presence of pneumonia virus. Forty animals, including 1 wild rat, were positive, i.e. 51 per cent. The presence of demonstrable virus appeared to be highly dependent on the age of the rats examined. Table 1 shows, that the virus has been recovered only from laboratory rats of at least 4 months of age.

We failed to produce experimental virus pneumonia in mice by subcutaneous, intraperitoneal, intramuscular, intravenous, and intracerebral inoculation, so that intranasal instillation seems to be the only route for infection. This route will presumably be the natural way by which the animals of a rat colony are infected. It was, however, difficult to induce contact infection in mice.

TABLE 1.

Isolation of pneumonia virus from laboratory rats of different age-groups.

| Age-group | Number of rats examined | Virologically positive |
|---|---|---|
| 2–15 weeks | 26 | 0 |
| 4– 6 months | 14 | 7 (50%) |
| 8–12 months | 18 | 15 (83%) |
| > 12 months | 18 | 17 (94%) |

Groups of 4 mice were kept in one cage together with 4 experimentally infected mice for periods of 18, 41, 90, and 150 days respectively. Typical lung lesions were found in only 1 out of the 4 mice that had been in contact with infected mice for a period of 150 days. Since we found demonstrable virus only in lungs of rats of at least 4 months of age, it might be assumed that in rats natural infection occurs only after prolonged contact. If this is so, separation of young rats in small groups as early as possible might be a suitable measure of preventing them from natural exposure.

Since suckling-mice proved highly susceptible to experimental infection, 9 female mice were infected during pregnancy, and the 70 animals of their offspring were sacrificed at ages varying from 7–60 days. Typical lung lesions were found in only 6 of them (8.6 %), that had been sacrificed at the age of 7, 10, 30, 32, and 60 days respectively.

## SOME PROPERTIES OF THE PNEUMONIA VIRUS.

Supernatant fluid of virulent mouse lung suspension retained its virulence after centrifuging for 30 minutes at 15.000 r.p.m. in a Spinco laboratory centrifuge. When centrifuged at 40.000 r.p.m. the supernatant fluid failed to produce virus pneumonia, whereas the resuspended sediment proved virulent. The virus particles, at least the bulk of them, can thus be sedimented at 40.000 r.p.m.

Electron micrographs of the virus in infected mouse lung suspension as partly purified by differential centrifugation and precipitation of normal lung components with celite and anti-mouse lung serum prepared in rabbits, a procedure described by GOGOLAK (4), revealed the presence of virus-like particles having a mean diameter of approximately 200–250 m$\mu$ (fig. 1). Such particles have not been found in similarly treated lungs from normal mice.

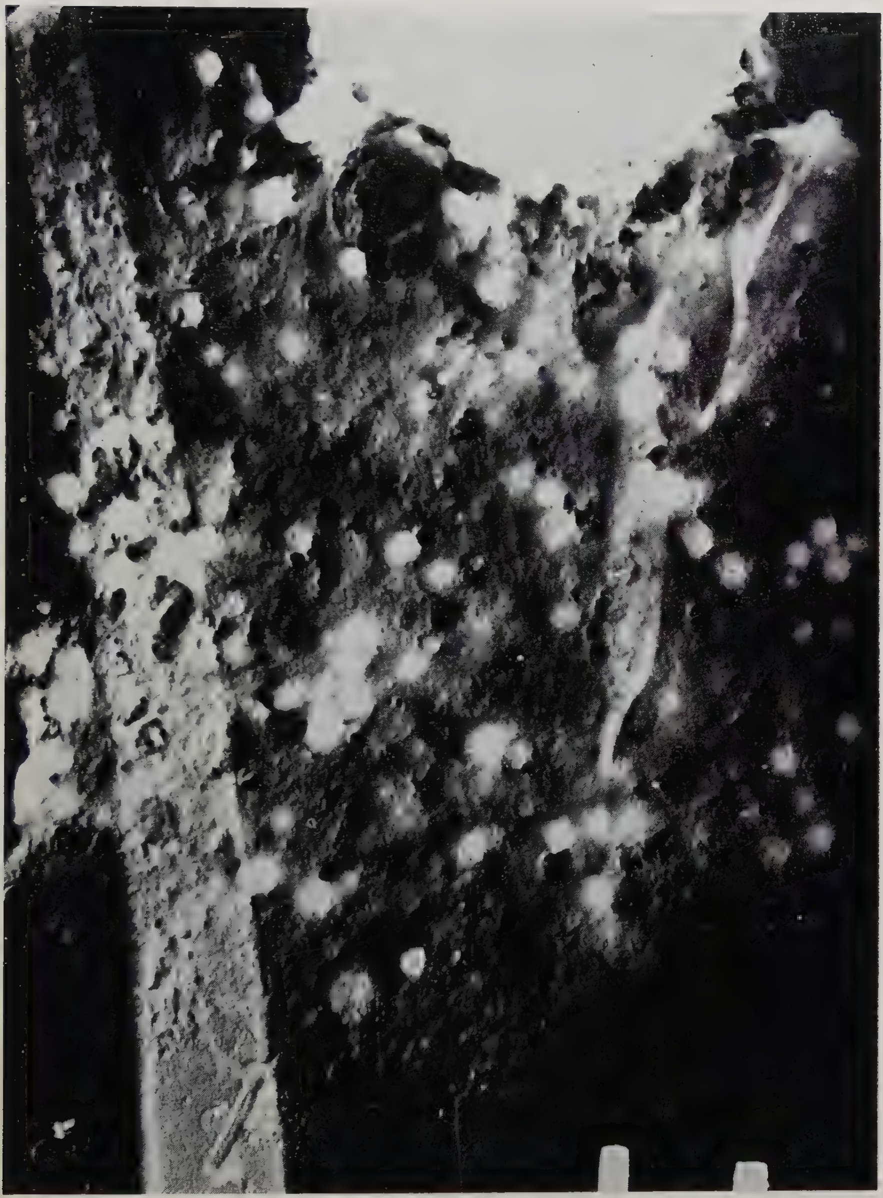

Fig. 1. Virus-like particles in infected mouse lung. Electron micrograph shadowed with palladium. Magnification 20.000 ×.

The virus passed through collodium membrane filters with an average pore diameter of 520 mμ. The filtration endpoint could not be determined accurately.

The virus remained virulent for several months when kept in dry-frozen lung at —20° C. The virulence was lost within 1 hour when kept in saline suspension at 37° C., at room temperature within a few hours, and at —20° C. within one week.

We failed to grow the virus in the chick-embryo following amniotic, allantoic, choriollantoic, and yolk sac inoculation, and in various tissue cultures (monkey testicle fibroblasts, human carcinoma cells of the "HeLa" strain, cells derived from transplantable mammary carcinoma of mice, embryonic human, mouse, and rat lung).

Virulent centrifuged mouse lung suspension failed to agglutinate mouse and fowl erythrocytes.

Attempts to produce neutralizing and complement-fixing antibodies by frequently repeated intramuscular inoculation of live virus suspension into rabbits, guinea pigs, and roosters failed. In naturally infected rats, and in experimentally infected mice, no neutralizing and complement-fixing antibodies against the rat pneumonia virus and against psittacosis virus could be shown.

### SENSITIVENESS TO CHEMOTHERAPEUTIC AND ANTIBIOTIC DRUGS.

Groups of 5 mice were infected with mouse passage virus by intranasal instillation, and treated with various chemotherapeutic and antibiotic agents for a period of 10 days, starting on the day of infection. The animals were sacrificed 14 days after infection, and the lungs were examined for lesions. The results are summarized in table 2, which shows the sensitiveness for both chlortetracyclin (aureomycin) and oxytetracyclin (terramycin).

The action of the antibiotics in this experiment is prophylactic rather than therapeutic. The virus could not be recovered from the lungs of mice that had received either chlortetracyclin or oxytetracyclin. It was, however, readily demonstrable in the lungs of the mice of all other groups.

In another experiment, groups of 5 mice received antibiotic treatment from the day of intranasal infection for different periods. All mice were sacrificed 14 days after infection. Table 3 shows, that chlortetracyclin is superior to oxytetracyclin, since the former prevents the development of lung lesions by treatment for 5 days

TABLE 2.

Sensitiveness of the rat pneumonia virus for chemotherapeutic and antibiotic agents.

| Drug | Daily dose | Administration | Lung lesions |
|---|---|---|---|
| Neosalvarsan | 1 × 1 mg | intraperitoneal | complete consolidation |
| Sulphamezathine | 3 × 2½ mg | ,, | ,, |
| Penicillin | 3 × 3000 U | subcutaneous | ,, |
| Streptomycin | 2 × 0.3 mg | ,, | ,, |
| Chloramphenicol | 2 × 2 mg | ,, | ,, |
| Chlortetracyclin | 2 × 1 mg | ,, | none |
| Oxytetracyclin | 2 × 1 mg | ,, | none |
| Controls | | | complete consolidation |

TABLE 3.

Influence of the duration of antibiotic treatment on the prevention of lung lesions and persistence of the virus.

| Antibiotic | Duration of treatment (days) | Lung lesions | Persistence of virus |
|---|---|---|---|
| Chlortetracyclin | 1 | ++++ | + |
| | 2 | ++++ | + |
| | 3 | ++ | + |
| | 4 | + | + |
| | 5 | — | + |
| | 6–10 | — | — |
| Oxytetracyclin | 1 | ++++ | + |
| | 2 | ++++ | + |
| | 3 | +++ | + |
| | 4 | + | + |
| | 5 | + | + |
| | 6 | + | + |
| | 7–10 | — | + |
| Chlortetracyclin and Oxytetracyclin | 1 | ++++ | + |
| | 2 | ++++ | + |
| | 3 | ++ | + |
| | 4 | ++ | + |
| | 5 | — | + |
| | 6 | — | + |
| | 7–10 | — | — |

or longer, and the virus is no longer demonstrable after treatment for 6 days or longer. With oxytetracyclin, treatment has to be continued at least 2 days longer in order to prevent the development of lung lesions, and the virus was shown to be active after treatment for as long as 10 days. No advantage of the combined action of both antibiotics was observed.

A third experiment was carried out to determine the therapeutic effect of chlortetracyclin and oxytetracyclin. In this experiment 36 mice were infected intranasally. Six animals were sacrificed on the 7th day after infection. They all showed typical lung lesions. The 30 remaining mice, that were expected to have the same lung lesions, were divided in 3 groups of 10 animals. One group was treated with chlortetracyclin, a second group was treated with oxytetracyclin, and the third group served as control. Treatment started on the 7th day after infection, and was continued for a period of 10 days. All mice were sacrificed 10 days after the treatment had been ceased.

Complete consolidation was found in all mice of the control group, whereas the animals of both treated groups showed no lung lesions at all.

When starting treatment with both antibiotics 27 days after intranasal infection for a period of 10 days, lung lesions were found in none of 10 mice that had received chlortetracyclin, and in 1 out of 10 mice that had received oxytetracyclin. The lungs of all 10 control mice showed complete consolidation. The animals had been sacrificed on the day after the last administration of the drug.

It may be concluded from these experiments, that both chlortetracyclin and oxytetracyclin have a marked therapeutic effect on experimentally induced virus pneumonia.

Finally, it has been examined as to whether antibiotic treatment, when started at a time that lung lesions had developed, had any effect on the activity of the virus.

Three groups of 25 mice received a daily dose of either 1 mg of chlortetracyclin or 1 mg of oxytetracyclin, or 0.5 mg of both of them from the 7th day after intranasal infection. Two mice from each of the groups were sacrificed at 1 day intervals, and 4 mice were infected with a suspension of their lungs. The presence of virus in the lungs of the treated mice was accepted when typical lung lesions were found in the mice infected with their lung material. The results were approximately the same as those recorded in table 3.

In the chlortetracyclin group no active virus was demonstrated after treatment for at least 5 days, and in the group treated with both antibiotics no active virus was demonstrated after treatment for at least 6 days. At that time, lung lesions were also absent. In the oxytetracyclin group, however, active virus was still present after treatment for 10 days, but no lung lesions were found.

The remaining mice were sacrificed 2 months after infection. Mice that had received chlortetracyclin, alone or in combination with oxytetracyclin did not show any lung lesions. Those, however, that had received oxytetracyclin alone showed again lung lesions. This experiment presents circumstantial evidence that chlortetracyclin inactivates the virus, and that oxytetracyclin fails to inactivate the virus.

### FAILURE TO PRODUCE ACTIVE IMMUNITY.

Two groups of 25 mice were treated with either chlortetracyclin, or oxytetracyclin, from the 7th day after intranasal infection for a period of 10 days. Five mice of each group were sacrificed immediately before and after treatment. All mice sacrificed previous to treatment showed lung lesions, but none were found in those sacrificed immediately after treatment. The 15 remaining animals of each group were reinfected intranasally 18 days after the first infection. They all showed typical lung lesions, which indicates that no active immunity had developed as a result of the first infection.

### RELATION OF THE PNEUMONIA VIRUS OF RATS TO GREY LUNG VIRUS OF MICE.

NELSON (12) described the pneumonia rate in a selected colony of albino rats as high as 52%. There was no pneumonia in young rats, 3–4 months of age. The disease induced in mice was characterized by pneumonia resembling that of the rat in its initial involvement: grey or pinkish-grey areas of consolidation, microscopically characterized by peribronchiolar lymphocytic infiltration, accumulations of polynuclear leucocytes, and compression of alveoli, which were filled with large mononuclear cells. The disease was transmissible to mice by intranasal instillation of lung suspension. Transmission by direct contact was less readily accomplished, and required a long exposure period. The virus was apparently of the same size as that of the pox viruses. It could not be grown in the chick-embryo.

This description highly agrees with our findings. A possible relation to grey lung virus of mice has been taken into consideration (14).

The grey lung virus has been discovered by Andrewes and Glover (1). This virus, which has been received through the courtesy of Dr C. H. Andrewes (National Institute for Medical Research, Mill Hill), also produces pinkish-grey areas of consolidation, and the histological lesions which have been described by Niven (15), are very similar to those found in the experimentally produced rat pneumonia in mice. The sensitiveness of the grey lung virus to chemotherapeutic and antibiotic agents (2), and the lack of active immunity following infection, are essentially the same as those observed in our studies on rat pneumonia. Moreover, a variant of grey lung virus has been recovered from lung lesions in cotton rats (3).

There are, as a whole, several arguments for assuming that the virus recovered from the rats in our investigation is closely related to grey lung virus of mice and cotton rats, and perhaps, to the virus of endemic pneumonia in albino rats. Since demonstrable antibodies and active immunity fail to develop, no evidence of this assumption can be given by immunological methods.

## Summary.

A pneumonia-producing virus in mice has been isolated from the lungs of 39 out of 76 white laboratory rats from different colonies, and of 1 out of 2 wild rats suffering from snuffling disease. The virus was transmissible only by intranasal instillation. Contact infection of mice occurred rarely and only after prolonged contact. Since only rats of at least 4 months of age appeared to be infected, contact infection in rats also may be difficult, and early separation of young rats in small groups might, perhaps, be an effective preventive measure. The experimentally induced disease in mice tends to be chronic, as it is in rats. The experimental disease in suckling-mice, however, was usually fatal. Electron micrographs of partly purified lung suspensions showed virus-like particles having a diameter of 200–250 mμ. Chlortetracyclin and oxytetracyclin have a prophylactic and a therapeutic effect on the experimental disease. Lesions disappear after antibiotic treatment. The virus seems to be inactivated only by chlortetracyclin. Mice that have recovered by anti-

biotic treatment proved not immune to reinfection. The properties of the virus are highly similar to those of grey lung virus of mice.

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# THREE NEW YEASTS

by

**J. P. VAN DER WALT** and **IRMGARD T. TSCHEUSCHNER**

(Received January 28, 1957).

Examination of eighteen yeast strains from soil and one from buttermilk, according to the standard methods of LODDER and KREGER-VAN RIJ (1952) has revealed the existence of three new species. Seventeen of the soil strains are gathered into one species for which the name *Saccharomyces lodderi* is proposed, while the remaining soil strain is classified as a *Candida* species, *viz.*, *Candida natalensis*. The strain from buttermilk has been designated as *Pichia pijperi*. The descriptions and Latin diagnosis of these species follow.

## *Saccharomyces lodderi* nov. spec.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are either short-oval to oval (2.5 – 7.0) × (3.1 – 8.0) $\mu$, single, in pairs or clusters; or cylindrical (1.6 – 4.0) × (3.5 – 12.0) $\mu$, single or in pairs. A sediment, slight ring and islets or pellicle are usually formed. After 1 month at 17°C., a sediment, ring or a thin pellicle and ring are usually formed.

Growth on malt agar: After 3 days at 25°C., the cells are of the same form and dimensions as in malt extract. Asci may be present. After 1 month at 17°C., the streak culture is cream-grey, somewhat shining with slight structure towards the centre. The margin is slightly undulating.

Slide culture: A primitive pseudomycelium, consisting of branched chains of elongated cells, is formed.

Sporulation¹): An isogamous or heterogamous conjugation

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¹) Spores are abundantly formed on malt agar and on all sporulation media.

usually precedes ascus formation: 1 – 4 spores are formed per ascus. The spores are long oval to reniform. They are soon liberated.

| | | | | |
|---|---|---|---|---|
| Fermentation: | Glucose | + | Maltose | — |
| | Galactose | + | Lactose | — |
| | Saccharose | + | Raffinose | $^1/_3$ |
| | Trehalose | + | | |

| | | | | |
|---|---|---|---|---|
| Sugar assimilation: | Glucose | + | Maltose | — |
| | Galactose | + | Lactose | — |
| | Saccharose | + | | |
| | Trehalose | + | | |

Assimilation of potassium nitrate: Absent.
Ethanol as source of carbon: Growth.
Splitting of arbutin: Absent.

## Discussion.

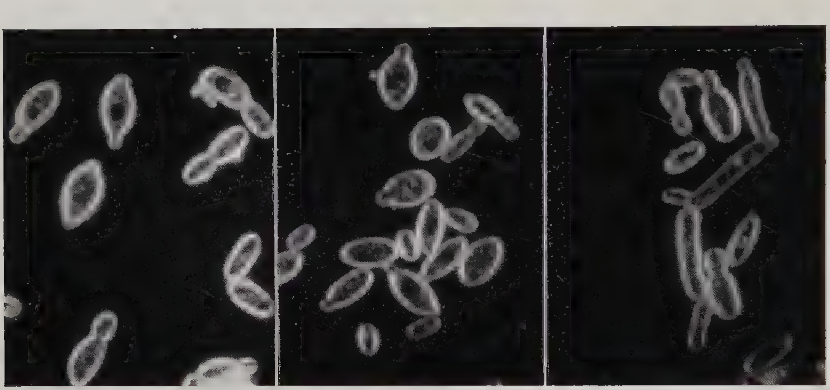
The 17 strains all of which were isolated from soil are provisionally brought to the genus *Saccharomyces* since they are strongly fermentative and do not assimilate nitrate. Since their long oval to reniform ascospores are soon liberated on the sporulation media, the strains, however, qualify for classification in the genus *Dekkeromyces*, recently proposed by Wickerham and Burton (1956). Biochemically the strains show a similarity with *Saccharomyces phaselosporus*, a species which similarly forms long oval to reniform ascospores and also ferments glucose, galactose, saccharose and raffinose for one third and does not assimilate maltose or lactose. Comparison with an authentic culture of *Saccharomyces phaselosporus*, however, (Shehata, Mrak and Phaff, 1955) showed distinct biochemical and cultural differences. All the strains from soil readily fermented trehalose while *Saccharomyces phaselosporus* failed to assimilate this sugar. On the other hand none of the strains from soil assimilated either sorbitol, mannitol or sorbose which were readily attacked by *Saccharomyces phaselosporus*. The most obvious cultural differences are that the strains from soil produce a primitive pseudomycelium and are able to grow at 37°C., neither of which *Saccharomyces phaselosporus* does.

Accordingly the strains are considered as representative of a new species. For this species the name *Saccharomyces lodderi* is proposed in honour of Dr J. Lodder in recognition of her classical contributions to yeast systematics.

## *Pichia pijperi* nov. spec.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are either oval to cylindrical (1.9 – 5.0) × (3.8 – 18.8) $\mu$, single, in pairs or short chains; or more or less apiculate, (1.9 – 6.0) × (3.8 – 10.3) $\mu$, single or in pairs. (Figs. 1,2 and 3). A dull thin creeping pellicle and slight sediment are formed. After 1 month a pellicle and sediment are formed.



Figs. 1, 2 and 3. *Pichia pijperi.* After 3 days in malt extract. (1,000 ×, sunlight darkfield microscopy).

Growth on malt agar: After 3 days at 25°C., the cells are either oval to cylindrical (1.4 – 4.4) × (3.1 – 16.0) $\mu$, single, in pairs or chains; or more or less apiculate (1.9 – 6.2) × (3.8 – 12.5) $\mu$, single or in pairs. After 1 month at 17°C., the streak culture is flat, dull, cream to greyish-white. The margin is lobate.

Slide cultures: Pseudomycelium of the "candida"-type is abundantly formed.

Sporulation[1]: No conjugation was observed. Mature spores are hat-shaped, 1 – 4 per ascus.

| | | | | |
|---|---|---|---|---|
| Fermentation: | Glucose | + | Maltose | — |
| | Galactose | — | Lactose | — |
| | Saccharose | — | | |
| Sugar assimilation: | Glucose | + | Maltose | — |
| | Galactose | — | Lactose | — |
| | Saccharose | — | | |

---

[1] Spores were observed on malt agar and on Gorodkowa agar.

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: Growth; a pellicle is formed.

Splitting of arbutin: Positive.

## DISCUSSION.

Although this strain has the rather exceptional property of forming more or less apiculate cells and budding generally bipolarly, it is classified as a *Pichia* species since it is also characterised by the early formation of a dull creeping pellicle and an abundant formation of pseudomycelium. Although resembling other members of the genus, *viz.*, *Pichia fermentans* and *Pichia terricola* (VAN DER WALT, 1957) in forming an early pellicle and fermenting only glucose yet not assimilating either galactose, saccharose, maltose or lactose, the strain from buttermilk differs in being able to assimilate mannitol, sorbitol, sorbose and cellobiose.

As the strain cannot be identified with any known organism, it must be considered as representative of a new species. For this species the name *Pichia pijperi* is proposed in honour of Professor dr A. PIJPER in recognition for his highly esteemed assistance to the authors.

## *Candida natalensis* nov. spec.

## DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are round, oval or cylindrical, (2.5 – 7.8) × (3.1 – 9.4) $\mu$, single, in pairs or in short chains. A dull creeping pellicle and sediment are formed. After 1 month a pellicle and sediment are present.

Growth on malt agar: After 3 days at 25°C., the cells are short oval to cylindrical, (1.6 – 5.6) × (2.5 – 10.6) $\mu$. After 1 month the streak culture is cream coloured, raised and rugose. The margin is fringed with pseudomycelium.

Slide cultures: A pseudomycelium of tree-like formation is abundantly formed.

Sporulation: No ascospores were observed.

Fermentation:

| | | | |
|---|---|---|---|
| Glucose | + | Maltose | — |
| Galactose | + | Lactose | — |
| Saccharose | + | (very weak) or | — |
| | | Raffinose | — |

| Sugar assimilation: | | | | |
|---|---|---|---|---|
| | Glucose | + | Maltose | + |
| | Galactose | + | Lactose | — |
| | Saccharose | + | | |

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: Growth; a pellicle is formed.

Splitting of arbutin: Absent.

## DISCUSSION.

As the strain forms a pseudomycelium and is apparently anascosporogenous it is provisionally classified as a *Candida* species. However, since WICKERHAM and BURTON have shown that some yeasts originally classified as anascosporogenous are haploid mating types, this strain from soil, if induced to sporulate by mating, would be included in the genus *Pichia*.

Since the properties of this strain do not correspond to any previously described member of the genera *Pichia* or *Candida*, it is proposed to name the species *Candida natalensis*, after the province from where it was first isolated.

## *Saccharomyces lodderi* nov. spec.

In musto maltato cellulae subovoideae ovoideaeque (2.5 – 7.0) × (3.1 – 8.0) $\mu$, et cylindricae (1.6 – 4.0) × (3.5 – 12.0) $\mu$, singulae aut binae. Sedimentum. Plerumque annulus et pellicula tenuis aut insulae formantur.

In agaro maltato formae et dimensiones cellularum eaedem sunt quae in musto maltato. Cultura (post unum mensem 17°C.) albiflava griseola, parum nitens plerumque cum structura subtili in parte media. Margine parum undulato.

Pseudomycelium formatur. Cellulae pseudomycelii et blastosporae longovoideae aut oblongae in catenis ramosis.

Copulatio cellularum aequarum inaequarumque conformationi asci plerumque praecedit. Ascosporae glabrae, longovoideae aut reniformes, 1 – 4 in asco. Ascosporae ex ascis celeriter liberantur.

Fermentatio glucosi, galactosi, sacchari, trehalosi et raffinosi pro tertia parte. In medio minerali cum glucoso, galactoso, saccharo et trehaloso crescitur. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico crescitur. Arbutinum non finditur.

Isolata ex terra.

## *Pichia pijperi* nov. spec.

In musto maltato cellulae longovoideae cylindricaeque (1.9 – 5.0) × (3.8 – 18.8) $\mu$, singulae, binae aut catenatae, aut cellulae fere apiculatae (1.9 – 6.0) × (3.8 – 10.3) $\mu$, singulae aut binae. Post dies 3 pellicula, non nitida sursum repens, formatur. Sedimentum.

In agaro maltato cellulae longovoideae cylindricaeque (1.4 – 4.4) × (3.1 – 16.0) $\mu$, singulae binae aut catenatae, aut cellulae fere apiculatae (1.9 – 6.2) × (3.8 – 12.5) $\mu$, singulae aut binae. Cultura (post unum mensem 17°C.) griseola albiflava, plana, non nitida. Margine paulum lobiformo.

Pseudomycelium abundat. Cellulae cylindricae in catenis ramosis.

Ascosporae maturae pileiformae; 1 – 4 in asco.

Fermentatio glucosi solius. In medio minerali cum glucoso solo crescitur. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico crescitur, pellicula formatur. Arbutinum finditur.

Isolata ex sero quod butyro facto e lacte secernitur.

## *Candida natalensis* nov. spec.

In musto maltato cellulae rotundae, aut cylindricae (2.5 – 7.5) × (3.1 – 9.4) $\mu$, singulae, binae aut catenatae. Post dies 3 pellicula, non nitida, sursum repens formatur. Sedimentum.

In agaro maltato cellulae subovoideae aut cylindricae (1.6 – 5.6) × (2.5 – 10.6) $\mu$, singulae, binae aut catenatae. Cultura (post unum mensem 17°C.) albiflava, acclivis et rugosa, margine piloso.

Pseudomycelium abundat. Cellulae cylindricae in catenis ramosis.

Fermentatio glucosi, galactosi et sacchari (valde exigua). In medio minerali cum glucoso, galactoso, saccharo et maltoso, crescitur. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico crescitur; pellicula formatur. Arbutinum non finditur.

Isolata ex terra.

## Acknowledgement.

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# A NEW *SALMONELLA* TYPE (*S. ANGOLA*)

by

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(Received April 12, 1957).

In January 1957 a culture isolated from fish-meal of Portuguese Angola origin was examined at the National Salmonella Centre.

This culture showed the morphological and biochemical characteristics of the genus *Salmonella*, e.g. Gram negative, motile rods, rapid fermentation of glucose (with gas), arabinose, rhamnose, trehalose, mannitol, dulcitol, sorbitol, xylose, citrate, mucate, d- and i-tartrate, production of $H_2S$, liquefaction of gelatin, reduction of nitrates. Indol was not formed, urea was not hydrolysed and salicin, inositol, sucrose, lactose, glycerol and i-tartrate were not fermented. Voges-Proskauer test negative, methylred test positive.

The organism was agglutinated to titre by *S. dublin* O serum; in absorption tests all the O-agglutinins were removed from this serum. A serum prepared with the new type agglutinated to the same titre the homologous strain and *S. dublin*. The O-agglutinins of this serum were all absorbed by *S. dublin*. The culture belonged therefore to group D of the genus *Salmonella*: O antigens 1, 9, 12.

The H antigens were diphasic, phase 1 was agglutinated to titre by *S. poona* serum (z) and phase 2 by serum *S. kentucky* (i; $z_6$). In absorbtion tests not all agglutinins of the *S. poona* serum were removed.

Dr KAUFFMANN told us that the z-antigen is a complex one, which has not be taken in account in the Kauffmann-White schema. Phase 2 absorbed all agglutinins of the *S. kentucky* serum $z_6$ phase, while H agglutinins of a phase 2 serum of the new type were all absorbed by *S. kentucky* $z_6$ phase. The antigenic formula of the type was therefore 1, 9, 12 : z : $z_6$. As a *Salmonella* type with this formula, is as yet not described, we propose the name *Salmonella angola*.

## Summary.

A new *Salmonella* type (*S. angola*) with the antigenic formula 1, 9, 12 : $z$ : $z_6$ is described.

We are indebted to Dr F. Kauffmann, International Salmonella Centre (Copenhagen), for the confirmation of our findings.

(Aus der Bundesstaatlichen bakteriologisch-serologischen Untersuchungsanstalt in Linz/Donau).

# ÜBER FLÜSSIGE TUBERKULOSENÄHRBÖDEN UND FLÜSSIGKEITSKULTUREN

bei

**A. MAREK**

(Eingegangen 2. Januar 1957).

Seit der Entdeckung des Tuberkuloseerregers durch R. Koch war eine Reihe von Forschern bemüht, die Züchtung des Tuberkelbazillus auf ein optimales Mass zu bringen. Nocard und Roux empfahlen einen Glyzerinzusatz zum Nährboden und Pawlowsky führte die Kartoffel als Nährboden ein. Capaldi war der erste, der Hühnerei zur Nährbodenpräparation vorschlug. Somit waren drei sehr wesentliche Grundsubstanzen für feste Kulturmedien gegeben, die mit kleineren Abweichungen von Löwenstein, Hohn, Petragnani u.a. zu den bekannten Nährböden variiert wurden. Es fehlte allerdings nicht an weiteren Versuchen, flüssige Nährmedien für die Möglichkeit des kulturellen Nachweises heranzuziehen. R. Koch verwendete noch Rinderserum und schlieszlich Glyzerinbouillon, allerdings mit der Absicht Tuberkulin in größeren Mengen herzustellen und nicht für die bakteriologische Diagnostik.

Im Laufe der Zeit wurde von zahlreichen Forschern eine grössere Anzahl flüssiger, synthetischer und halbsynthetischer Nährmedien vorgeschlagen (siehe Tabelle 1). Auch Nährmedien mit Vollblut oder Zitratblut wurden von Wright (21) eingeführt, von Meissner (11) modifiziert und in Anlehnung an diese grundlegenden Arbeiten von Langenscheidt (9) in den letzten Jahren, speziell auf dem Sektor der Veterinärmedizin erfolgreich angewendet. Sauton (17) erbrachte den Beweis, wie wichtig Eisen für das gute Wachstum des Tuberkuloseerregers ist, da es schon in einer Verdünnung von 1 : 100.000 zugesetzt, nahezu die dreifache Bakterienmenge hervorzubringen imstande ist. Besredka (2) empfahl gemeinsam mit Jupille vor-

TABE

| | 1894 Proskauer-Beck | 1909 Miehe | Dorset | 1911 Lockemann | 1912 Sauton | 1923 Frouin Guillaume | 1949 Darzin | 1949 Hermann | Substrat 20 Goebel |
|---|---|---|---|---|---|---|---|---|---|
| $KH_2PO_4$ | 5 | | | 4 | | 1 | 1 | 1 | 1 |
| $K_2HPO_4$ | | 1 | 1.8 | | 0.5 | | | | |
| $NaH_2PO_4$ | | | | 3 | | | | | |
| $Na_2HPO_4$ | | | | | | | 5 | 4.5 | 9 |
| $MgSO_4$ | 0.6 | 0.2 | 1.5 | 2.5 | 0.5 | 1 | 0.2 | 0.6 | 0.6 |
| Mg-citrat | 2.5 | | | | | | | | |
| Na-citrat | | | 0.9 | 2.5 | | 1 | | 3 | 3 |
| Acidum citrium | | | | | 2 | | | | |
| Ferricitrat | | | 0.3 | | | | | | |
| Ferri-ammoniumcitrat | | | | | 0.05 | | | | |
| Ferri-ammoniumsulfat | | | | 0.1 | | | | 0.1 | 0.01 |
| Asparagin | 5 | 5 | 12 | 5 | 4 | 5 | 2 | 3 | 3 |
| Alanin | | | | | | | | | |
| Dextrose | | 40 | 10 | | | | | | |
| $NH_4Cl$ | | | | | | | | 5 | 5 |
| $NH_4$ citrat | | | | | | | | | |
| $CaCl_2$ | | 0.1 | | | | | | | |
| Glyzerin | 20 | | 100 | 25 | 60 | 40 | 20 | 20 | 20 |
| $ZnSO_4$ | | | | | | | | | |
| $CuSO_4$ | | | | | | | | | |
| Casein | | | | | | | | | |
| Tween 80 | | | | | | | | | |
| Albumin (bovine-plasma Fraktion V) | | | | | | | | | |
| Pepton | | | | | | | 5 | | |
| Rindervollblut | | | | | | | | | |
| NaCl | | | | | | | | | |
| $Na_2CO_3$ | | | | | | | | | |
| Aqua dest. | | | | | | | | | |

erst die Eibouillon (Gesamtei), verwendete aber wegen der Hemmwirkung des Eiweisses 20-fach verdünnte reine Dottersubstanz. WOLTERS (20) hat unter Abänderung die Eigelbbouillon empfohlen und BOECKER (3) verwendete erfolgreich eine Glyzerinbouillon mit 1,3 % Dottersubstanz. Besonders hervorgehoben seien die von KIRCHNER (7) angegebenen synthetischen Nährlösungen, die in geringer Weise umgewandelt, SULA (19) für seine Modifikation sicherlich als Grundlage dienten. KIRCHNER hat 3 Nährlösungen vorgeschlagen:

1

| Unholtz-Gaubatz | Sula | Dubos | Long-Siebert | Kirchner Sy-Ser | Kirchner Min-Ser | Kirchner Min-As-Ser | Langenscheidt |
|---|---|---|---|---|---|---|---|
| 4 | 1.5 | 1 | 3 | 4 | 4 | 4 | |
| | | | | 3 | 3 | 3 | |
| 3 | 2.5 | 6.3 | | | | | |
| 0.6 | 0.5 | 0.01 | 1 | 0.6 | 0.6 | 0.6 | |
| 2.5 | 1.5 | 2.5 | | 2.5 | 2.5 | 2.5 | 30.0 |
| | 0.05 | 0.005 | 0.05 | | | | 2 |
| 0.5 | | | | | | | |
| 5 | 2.5 | 1 | 5 | 5 | | 0.5 | |
| 2 | 0.15 | | | | | | |
| | | | | | | | 10.0 |
| | | | 5 | | | | |
| | | 0.0005 | | | | | |
| 20 | 30 | | 50 | 20 | | | |
| | | 0.0001 | | | | | |
| | | 0.0001 | | | | | |
| | | 1 | | | | | |
| | | 100 | | | | | |
| | | | | | | | 300 ml |
| | | | 2 | | | | |
| | | | 1 | | | | |
| | | | | | | | 700 ml |

1. Die synthetische Nährflüssigkeit mit 10 % Serumzusatz (SY-SER).
2. Die Mineralsalzlösung (ohne Asparagin und Glyzerin) mit 10 % Serumzusatz (MIN-SER).
3. Die Mineralsalz-Asparagin-Lösung mit 10 % Serumzusatz (MIN-AS-SER).

Sula fügte zur synthetischen Nährflüssigkeit „SY-SER" noch Alanin und Eisenammoncitrat hinzu, sodass sich nun vergleichend folgende Zusammensetzung ergibt:

TABELLE 2.

| Ingredientien | SULA-Medium | SY-SER | MIN-SER | MIN-AS-SER |
|---|---|---|---|---|
| $Na_2HPO_4$ | 2,5 | 3,0 | 3,0 | 3,0 |
| $KH_2PO_4$ | 1,5 | 4,0 | 4,0 | 4,0 |
| $MgSO_4$ | 0,5 | 0,6 | 0,6 | 0,6 |
| Na-Citrat | 1,5 | 2,5 | 2,5 | 2,5 |
| Asparagin | 2,5 | 5,0 | — | 0,5 |
| Alanin | 0,15 | — | — | — |
| Fe-Ammoniumcitrat | 0,05 | — | — | — |
| Glyzerin | 30,0 | 20,0 | — | — |

Kirchner verwendete Humanserum, während für unseren Gebrauch sich das Rinderserum im Sula-Medium gut bewährt hat.

Die einfache und rasche Herstellung des flüssigen Nährmediums nach Sula hat uns bewogen, dieses Substrat nicht nur für die Empfindlichkeitsprüfungen der Tuberkelbakterien nach der Methode Müller-Price-Jensen-Dissmann-Iglauer (4, 6, 12, 14) zu verwenden, sondern auch für die diagnostische Züchtung aus geringen Untersuchungsmaterialmengen heranzuziehen.

Gottsacker empfiehlt allerdings nicht, den flüssigen Nährboden an Stelle eines Eiernährbodens zu verwenden, besonders wenn es sich um diagnostische Untersuchungen handelt. Leider ist das vorliegende, uns zur Verfügung stehende Untersuchungsmaterial nicht immer in der gewünschten Menge vorhanden, sodaß wir uns genötigt sahen, auch flüssige Nährmedien für diagnostische Kulturen (Vorkulturen) heranzuziehen. Besonders vorteilhaft hat sich die Methode der Flüssigkeitskultur bei Larynx-Abstrichen, bei Abstrichen aus tuberkulösen Fisteln und bei Sekretabsaugungen aus Bronchialstümpfen nach Lobektomien erwiesen. Die Larynxabstriche wurden besonders von den Auswanderungskommissionen angefordert, weil damit eine Sicherstellung der Identität des Probanden gegeben war und einer Unterschiebung jederzeit vorgebeugt werden konnte. Klinisch ergab sich bei diesem Personenkreis oft gar kein Anhaltspunkt für eine spezifische Affektion, oder es konnten alte verkalkte Residuen einer abgeheilten Tuberkulose röntgenologisch gefunden werden. Daraus erklären sich auch zwanglos die überwiegend negativen Kulturergebnisse bei Larynxabstrichen unseres Untersuchungsmateriales.

## EIGENE UNTERSUCHUNGEN.

Die bisherigen Beobachtungen und Erfahrungen wurden an 306 Flüssigkeitskulturen gemacht, die uns ermutigen, von dieser Verarbeitungsmethode reichlicher Gebrauch zu machen. Bis jetzt konnten wir 27 positive Kulturen vermerken, unter denen 2 Kehlkopfabstriche, 18 Fistelabstriche und 7 Bronchialsekretabsaugungen vorhanden waren.

Die parallel hiezu angelegten Löwensteinkulturen ergaben eine Differenz bei den Kehlkopfabstrichen. Sie blieben negativ. Dies könnte damit erklärt werden, dass das spärliche auf den Abstrichen befindliche Sekret durch die Vorbehandlungs- und Waschprozeduren nicht unerheblich reduziert werden dürfte, was die Chancen auf einen positiven Kulturerfolg geringer werden läßt. Eine weitere Verminderung der Erfolgsaussichten wird zu erwarten sein, wenn das vorbehandelte Sekret auf einen festen Nährboden ausgestrichen wird. Wenn nicht zufällig die wenigen Tuberkelbakterien an der Nährbodenoberfläche haften, wird die Kultur steril bleiben.

Anders liegen die Verhältnisse bei flüssigen Nährmedien, in denen die einzelnen Tuberkelbakterien aufgeschwemmt, geeignete Bedingungen vorfinden, um sich vermehren zu können. Aus dem Sediment dieser Flüssigkeitskulturen ist der mikroskopische Nachweis nach 8–10 Tagen kein Problem und gibt außerdem noch die Möglichkeit, eine Löwensteinkultur anzulegen, um säurefeste Saprophyten ausschließen zu können. Die allgemeinen Wachstumstendenzen werden durch die flüssige Vorkultur begünstigt, sodass nach Überimpfung auf Löwensteinnährböden schon nach 14 Tagen eine makroskopisch deutliche Koloniebildung beobachtet werden kann. Da säurefeste Saprophyten a priori von pathogenen Stämmen nicht leicht differenziert werden können, haben wir die Sekundärkultur auch auf gewöhnlichem Nähragar bei 37° C. und bei Zimmertemperatur (etwa 22° C.) vorgenommen. Auf diese Weise hatten wir eine brauchbare Diffenzierung.

In Anlehnung an die von ABBOT (1) angegebene Vorbehandlung des Untersuchungsmateriales, haben wir die Abstriche und Tupfer mit einer Penicillinlösung, die in 1 ml 5000 IE gelöst enthielt, verarbeitet. Das Untersuchungsmaterial auf dem Tupfer verblieb 20–30 Minuten in dieser Penicillinlösung und wurde danach sofort ohne weitere Präparation in das flüssige Nährmedium von SULA übertragen. Eine sekundäre Verunreinigung des SULA-Mediums konnte praktisch bei keiner einzigen von 306 Chargen beobachtet werden.

## BEOBACHTUNGEN ANDERER AUTOREN

KÜNZEL (8) hat 1950/51 über vergleichende Untersuchungen zwischen dem SULA-Medium und der nach PETRAGNANI hergestellten Eiernährböden berichtet und war an Hand von 600 Kulturen zu dem Schluss gekommen, daß mit Hilfe eines flüssigen Nährmediums zusätzlich eine nahezu 40–50 %-ige Mehrausbeute an positiven Befunden zu erwarten ist, als auf festen Kulturmedien allein. Die optimale Methode liegt nach seinen Angaben bei der Anwendung eines flüssigen und eines festen Nährbodens. POTHMANN (13) teilt ebenso seine Erfahrungen mit flüssigen Tuberkulosenährböden mit. Aus 600 verschiedenartigen Materialeinsendungen konnten 123 Tuberkelbakterienstämme isoliert werden. Seiner Ansicht nach kann die Ausbeute positiver Kulturen mittels flüssiger Medien um etwa 20 % gesteigert werden. Die Untersuchungsergebnisse von KÜNZEL, POTHMANN und die eigenen Zahlen sind in Tabelle 3 zusammengestellt.

TABELLE 3.

| Autoren | Zahl der Unters. | Summe der posit. Kulturen | Positive Kulturen | | |
|---|---|---|---|---|---|
| | | | auf festen Eiernähr. Medium | nur in flüss. Med. | auf beiden Nährmedien |
| KÜNZEL | 600 | 93<br>(15,5%) | 20<br>(21,5%) | 41<br>(44,1%) | 32<br>(34,4%) |
| POTHMANN | 600 | 123<br>(20,5%) | 40<br>(32,5%) | 25<br>(20,3%) | 58<br>(47,2%) |
| eigene Unters. | 306 | 27<br>(8,82%) | — | 2<br>(7,45%) | 25<br>(92,55%) |

Skandinavische Autoren haben sich eingehend mit den Flüssigkeitskulturen beschäftigt. SMEDSRUD (18) kam bei seinen vergleichenden Untersuchungen zu dem Schluss, daß der Erfolg von der reichlichen Tuberkelbakterienanzahl im Untersuchungsmaterial und deren regelmässige Ausscheidung abhängig ist. GILJE (5) empfiehlt die Larynxkultur der Magenspülflüssigkeitskultur absolut vorzuziehen. Die Zuverlässigkeit der Larynxkultur liegt nach RAMBØL (15) unter anderem in der sofortigen Verarbeitung des Untersuchungsmateriales. Tagelanges Aufbewahren vermindert die Ausbeute positiver Kulturen um 44 % bis 62 %.

SAENZ (16) sieht im Ausstrichverfahren und in der flüssigen Kulturmethode ein mindestens gleichwertiges, wenn nicht überlegenes Verfahren im Vergleich zur üblich geübten Kulturtechnik auf festen Nährböden. Er hebt besonders die Vorteile der Flüssigkeitskultur – die Einfachheit, Schnelligkeit und die geringe Verunreinigungsmöglichkeit – hervor.

Was uns jedoch im Verlauf von 3 Jahren bei der Präparation des SULA-Mediums aufgefallen ist, liegt in der Erkenntnis der Reifung des fertiggestellten Nährmediums. Unsere Beobachtungen haben nämlich gezeigt, daß ein und derselbe Nährboden sofort nach der Fertigstellung ein sehr schlechtes Tbc-Wachstum erkennen liess, während nach einer Reifungszeit von 2–3 Wochen dieser anfangs schlechte Nährboden von keinem anderen besser ersetzt werden konnte. Fraktionierte Untersuchungen im Verlauf einer 3-wöchigen Reifungsperiode haben ergaben, dass gutes Wachstum schon in der 2. Woche, jedoch sehr starkes Tuberkelbakterienwachstum in der 3. Woche zu verzeichnen ist. In einer früheren Veröffentlichung wurde vom Verfasser (10) auf die Qualität verschiedener Rinderseren hingewiesen, die durch entsprechende Vorversuche als geeignet oder ungeeignet für das halbsynthetische Medium von SULA angesehen worden waren. Versager bei den Wachstumskontrollen, wie sie anfänglich vorgekommen sind, konnten durch die Reifung des fertigen Nährbodens für 3 Wochen bis auf Einzelchargen zum Verschwinden gebracht werden. Das verwendete Rinderserum liess während der Reifungszeit weder Ausflockungen, noch sonstige Dehydratationserscheinungen erkennen. Die Einstellung des Nährmediums erfolgte auf einen pH-Wert von 6,8, der während der Reifung laufend kontrolliert, so gut wie konstant blieb.

Für den Sektor der Veterinärmedizin hat LANGENSCHEIDT (l.c.) einen flüssigen Tuberkulose-Nährboden bekanntgegeben, von dem folgende interessante Tatsache kurz festgehalten sei: Es handelt sich um ein Rinderblutmedium, welches 1–3 Tage nach der Fertigstellung für das Routineverfahren schlecht brauchbar war. Erst durch die weitere „Reifung" bis zum 15. Tag nach der Fertigstellung wurde die optimale Leistung und Sicherheit des Nährmediums erreicht. Danach wurde ein zunehmender Abfall der Leistungsfähigkeit beobachtet. Die Ursache des Steigens und Sinkens der Nährbodengüte ist nach Angaben des genannten Autors unbekannt.

Abschliessend sei gesondert vermerkt, daß die Aufklärung der biochemischen Vorgänge im Nährmedium während des Reifungsvorganges derzeit Gegenstand weiterer Versuche darstellt.

## Zusammenfassung.

Die Erfahrungen mit dem flüssigen Nährsubstrat von Sula werden bei der primären Züchtung von Tuberkelbakterien aus sterilem oder verunreinigtem Untersuchungsmaterial als günstig angesehen. Besonders bei Larynx- oder Fistelabstrichen wird die Flüssigkeitskultur als ,,Vorkultur'' bevorzugt. Ebenso vorteilhaft hat sich die Präparation der Abstriche mit einer Penicillinlösung an Stelle der Schwefelsäure erwiesen, ohne dass hierbei eine Schädigung des Nährsubstrates, oder eine verminderte Ausbeute positiver Kulturen beobachtet worden wäre. Die Qualität des Nährsubstrates ist zum Teil vom verwendeten Rinderserum abhängig, lässt sich aber durch eine 3-wöchige ,,Reifung'' im Kühlschrank erheblich verbessern. Die bisher gemachten Erfahrungen beruhen auf einer 3-jährigen Beobachtung.

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# THE APPLICATION OF A PROTEIN FRACTION DERIVED FROM *TREPONEMA PALLIDUM* (REITER STRAIN) AS AN ANTIGEN IN THE SERODIAGNOSIS OF SYPHILIS

by

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(Received October 29, 1956).

## INTRODUCTION.

The use of a phenolized suspension of the avirulent cultivable Reiter strain of *Treponema pallidum* as an antigen in the complement fixation test for syphilis was first recommended by GAEHTGENS (1929). Among many others, ERICKSON and EAGLE (1940) reported this antigen ('Palligen') to compare favourably in both sensitivity and specificity with alcoholic tissue-extracts. These facts, however, fell into the background when cardiolipin was discovered by PANGBORN (1941). The introduction of mixtures of the chemically defined substances: cardiolipin, lecithin, and cholesterol, as antigens, though facilitating the standardization of the serodiagnosis of syphilis, did not solve the problem of the 'biologic false positive' reactions. Thereupon the *Treponema pallidum* immobilization (TPI) test was presented by NELSON and MAYER (1949). Leaving out of consideration the other tests employing virulent treponemes as an antigenic source (TPIA, TPA and TPCF), which are still in the experimental stage, the TPI test is generally accepted to be the most specific in the serodiagnosis of syphilis. Because of the special requirements, its application, however, is restricted to a relatively small number of laboratories.

Recently attention has again been drawn to the excellent antigenic properties of phenolized suspensions of the Reiter treponema in the complement fixation test by PAUTRIZEL, SZERSNOVICZ and GIMENEZ (1953), BÉNAZET, BROTTES, THIVOLET and SOHIER (1954), Hardy, Bornand and Durel (1955), Mutermilch and Delaville (1955), Gastinel, Vaisman and Hamelin (1956).

In searching after the origin of the reactivity between the Reiter treponema and human syphilitic serum, the preliminary investigations of Beck (1939), Eagle and Hogan (1940) were confirmed and extended by D'Alessandro, Oddo and Dardanoni (1950). They revealed the Reiter treponema to contain four different fractions:

1. A lipoidal fraction, serologically related to alcoholic extract of beef heart. As absorption of the corresponding antibody (reagin) from syphilitic serum did not alter the reactivity between suspensions of the Reiter treponema and the serum, the lipid seems to be situated in such a way as to be inactive in the complement fixation test.
2. A lipoidal fraction, serologically related to alcoholic extract of beef brain. In syphilitic serum there is no corresponding antibody.
3. A polysaccharide fraction, serologically inactive with syphilitic serum.
4. A protein fraction, possessing a corresponding antibody in syphilitic serum, as appeared from its reactivity in the complement fixation test. The preliminary results obtained by D'Alessandro and Dardanoni (1953) with this fraction, which will here be referred to as protein antigen, led to the study of its practical significance beside cardiolipin antigen in the complement fixation test in comparison with the TPI test.

## Materials and methods.

The cultivation of the Reiter treponema and the preparation of the protein antigen were performed according to the directions given by D'Alessandro and Dardanoni (1953).

### Cultivation of the Reiter treponema.

The Reiter strain of *Treponema pallidum* was cultured in Brewer thioglycollate medium ('Difco'). In order to remove the agar contained, the dehydrated medium was dissolved in cold distilled water (40.5 g/l), refrigerated overnight and filtered. After autoclaving at 120°C. for 20 minutes and cooling, 10% rabbit serum, previously heated at 58°C. for 2 hours, was added. The culture medium, in 500 ml rubber capped flasks, was inoculated and incubated at 37°C. for 7 days under occasional shaking. At the end of this period the organisms were separated by centrifuging, washed twice and finally suspended in saline (1 g of moist sediment in 20 ml).

### Preparation of the protein antigen.

The suspension was submitted to cryolysis. The freezing (in solid $CO_2$-alcohol mixture) and thawing (in 37°C. waterbath) procedure was repeated 10 times. Subsequently the suspension was centrifuged, the supernatant saved, and the sediment suspended in half the original quantity of fresh saline. The whole procedure was repeated 4 times, yielding 60 ml lysate for each gram of the original moist sediment.

The lysate was placed in a cellophane bag and dialysed at 5°C. against 600 ml of ammonium sulfate solutions with increasing concentrations, previously adjusted to pH 7.4 with ammonium hydroxide. The dialysis was allowed to take place under continuous stirring for 12 hours with 10%, 20%, 30% and 40% saturated solutions and for 24 hours with 50% and 75% saturated solutions respectively. The lysate, decreasing in volume, became more and more opalescent. Finally a mucous precipitate developed.

The contents of the cellophane bag were transferred to a centrifuge tube and kept at 5°C. overnight. After centrifuging, the supernatant was discarded and the precipitate dissolved in 10 ml veronal-buffered saline (pH 7.4). The slightly turbid solution was dialysed against several portions of fresh solvent until ammonium sulfate was no longer detectable. Finally the dialysed solution was centrifuged at high speed, the sediment discarded and the supernatant preserved with 'Merthiolate' (1 : 10000).

### Complement fixation technique.

The complement fixation test (CFT) used was a highly standardized routine technique¹). As in the case of cardiolipin antigen, the reaction between protein antigen and syphilitic sera usually showed a marked prozone phenomenon (table 1). The optimal antigen dilutions employed in the CFT were: cardiolipin antigen 1 : 200 and protein antigen 1 : 80. Even undiluted protein antigen was devoid of anticomplementary activity.

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¹) To be published.

TABLE 1.

Titration of protein antigen.

| Serum dilutions | Antigen dilutions 1 : 5 | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | 1 : 160 | 1 : 320 |
|---|---|---|---|---|---|---|---|
| 1 : 5 | +++ | ++++ | ++++ | ++++ | +++ | + | — |
| 1 : 10 | — | + | +++ | +++ | +++ | + | — |
| 1 : 20 | — | — | — | — | + | — | — |
| 1 : 40 | — | — | — | — | — | — | — |

TABLE 2.

Comparison between the CFT with protein antigen and the TPI test.

| TPI | CFT with protein antigen | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | + | | | | | | — | | | | | |
| + | 107 | | | | | | 3 | | | | | |
| | *CFT with card. ant.* | | | | | | *CFT with card. ant.* | | | | | |
| | + | | | — | | | + | | | — | | |
| | 96 | | | 11 | | | 3 | | | 0 | | |
| | | | | *clinically* | | | *clinically* | | | | | |
| | | | | + | ? | — | + | ? | — | | | |
| | | | | 8 | 2 | 1 | 2 | 1 | 0 | | | |
| — | 16 | | | | | | 134 | | | | | |
| | *CFT with card. ant.* | | | | | | *CFT with card. ant.* | | | | | |
| | + | | | — | | | + | | | — | | |
| | 6 | | | 10 | | | 23 | | | 111 | | |
| | *clinically* | | | *clinically* | | | *clinically* | | | | | |
| | + | ? | — | + | ? | — | + | ? | — | | | |
| | 2 | 2 | 2 | 8 | 0 | 2 | 0 | 2 | 21 | | | |

## RESULTS.

A number of sera, forwarded to our Institute for the TPI test, were in addition subjected simultaneously to the CFT with protein antigen and to that with cardiolipin antigen. Sera which gave inconclusive results in one or more of these tests were left out of consideration. The results obtained are summarized in table 2.

Out of a total of 260 sera, 116 were considered to be syphilitic, either because all tests were positive or on account of the clinical and anamnestic data, 137 were classified as non-syphilitic, either because of the negativity of all tests or in view of the clinical and anamnestic data, while the available data of 7 serologically discrepant sera did not allow of a diagnosis.

Sensitivity: Of the 116 syphilitic sera, 114 were revealed by the CFT with protein antigen, 106 by the TPI test and 100 by the CFT with cardiolipin antigen. The CFT with protein antigen thus exhibited the highest sensitivity of the three tests under consideration.

Specificity: When applied to the 137 presumably non-syphilitic sera, the TPI test gave 1, the CFT with protein antigen 5, and the CFT with cardiolipin antigen 23 positive reactions respectively. The specificity of the CFT with protein antigen though being less than that of the TPI test, proved to be superior to that of the CFT with cardiolipin antigen.

In view of these facts, it is suggested that the CFT with protein antigen is a valuable verification procedure, which can be performed in every routine laboratory, whenever a discrepancy is observed between the results obtained with the standard serological tests and the clinical data.

The influence of therapy on the disappearance of antibodies from syphilitic serum depends on the stage of the illness at which treatment is started. Generally the CFT with protein antigen tends to remain positive for a long time.

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## Summary.

A comparison was made between the complement fixation test using a protein fraction derived from *Treponema pallidum* (Reiter strain) as an antigen and the *Treponema pallidum* immobilization test. As appears from the results obtained with 116 syphilitic and 137 presumably non-syphilitic sera the complement fixation test with protein antigen showed an excellent sensitivity together with a satisfactory specificity.

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# THE OCCURRENCE OF *SALMONELLA* AMONG ANIMALS IN ARUBA (NETHERLANDS ANTILLES)

by

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(Received March 21, 1957).

## A. INTRODUCTION.

In 1954 and 1955 there were repeated cases of slaughter cattle, imported into Aruba from Central America, dying in transit, while even those animals which succeeded in surviving the journey were invariably in such poor condition on arrival that large numbers of them subsequently died after disembarking. Investigations soon narrowed down the cause to a *Salmonella* infection. This led to a regular control of the occurrence of *Salmonella* carriers among the various imported slaughter cattle on one hand and, on the other, to a similar investigation embracing the indigenous cattle, sheep, goats and pigs of Aruba, as well as small domestic animals, especially dogs and cats. A preliminary investigation covering other potential sources of infection was also initiated in conjunction herewith.

## B. LITERATURE.

The first case in man in the Netherlands Antilles was reported in St. Eustatius in 1933. The causative *Salmonella* species, however, was not typed on this occasion. During the course of the following years the number of faeces specimens examined increased considerably, while the number of positive reactions likewise increased both absolutely as well as relatively.

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Important contributions were made by FELSENFELD and YOUNG in 1949 and 1952. The results hereof, together with the data abstracted from the official annual reports of the Netherlands Antilles for the years 1953–1955, are incorporated in Table 6. Although important data on the occurrence of *Salmonella* in man are to be found in these annual reports, data on animals are extremely scarce. Mention of *Salmonella* in animals was only found in the annual report for 1940–1941, which gives the following details:

S. *typhi murium*, in cattle, once from the spleen and a lymph gland and twice from the intestine.

S. *cholerae suis*, in two pigs, once from the flesh and once from a lymph gland.

GUILBRIDE (1953) conducted a survey of animal diseases in the Caribbean area of importance from a public health point of view. The data regarding the occurrence of *Salmonella*, incorporated in Table 1, are taken from this survey. This may be complemented with data in respect of Puerto Rico, i.e. that 53.5 % of the rats gave a positive result. The corresponding figures for freshly slaughtered pigs and chickens were 40 % and 14 % respectively.

In Mexico, VARELA and ZOZAYA (1941) examined 100 dogs in Mexico City and found 9 *Salmonella* hosts (S. *new brunswick* 2 ×, S. *give* 2 ×, S. *cerro* 1 ×, S. *montevideo* 2 ×, S. *derby* 1 × and S. *meleagridis* 1 ×).

Finally, mention must be made of the fact that S. *enteritidis*, S. *gallinarum* and S. *typhi suis* were reported from animals in the Dominican Republic (GUILBRIDE 1953).

The foregoing literature data thus corroborate the fact that numerous *Salmonella* types occur both in man and animals in the Caribbean area.

## C. OWN INVESTIGATIONS.

### 1. Technique.

For the collection of material destined for bacteriological examination, sterilized throat swabs were employed. After being passed over the intestinal section under examination, this swab was deposited in a sterile tube, that part of the wooden stick in contact with the fingers being broken off on the edge of the tube. The nutrient medium was then poured into the tube under appropriate aseptic conditions.

TABLE 1.

The occurrence of *Salmonella* in certain countries in the Carribbean area.

| | Curaçao 1) man | Jamaica 1) | | Costa Rica 1) man | Puerto Rico 2) | | Colombia 3), Venezuela man |
|---|---|---|---|---|---|---|---|
| | | man | animal | | man | animal | |
| *S. paratyphi* A | | | | | | | 2 |
| *S. paratyphi* B | 20 | | | | 1 | 2 Pi | 8 |
| *S. typhi murium* | 12 | 23 | 1/M<br>1/Ch<br>4/P | 5 | 10 | 5 R | 38 |
| *S. reading* | | | | | 1 | | 2 |
| *S. derby* | 3 | | | | 2 | 2R/7Pi | 6 |
| *S. bredeney* | 1 | | | 1 | | | 1 |
| *S. abortus ovis* | | | | | 1 | | |
| *S. cholerae-suis* | 1 | | | | | 6 Pi<br>3 P 1B | 6 |
| *S. thompson* | | | | | | | 1 |
| *S. oranienburg* | 7 | 1 | | | 1 | 2R/1Pi/2P<br>2 Ch | 7 |
| *S. bareilly* | | | | | | | 3 |
| *S. montevideo* | 1 | 8 | 1 Ch/2 P | | 1 | | 6 |
| *S. newport* | 5 | 6 | 1 P | 3 | 2 | 2R/4Pi/2P<br>1 Ch | 12 |
| *S. morbificans-bovis* | | | | | | | 1 |
| *S. typhosa* | 1 | 13 | | 1 | | | 26 |
| *S. enteritidis* | 26 | 2 | | | | | 4 |
| *S. dublin* | | 5 | 2C/3B/2M | | | 1R/1P | 4 |
| *S. panama* | 5 | 1 | | | 2 | | 9 |
| *S. london* | | | | | | | 2 |
| *S. give* | 2 | | 1 P | | | | 3 |
| *S. anatum* | 2 | 4 | 3 P | 1 | | 1 P | 14 |
| *S. new brunswick* | | | | | | | 1 |
| *S. senftenberg* | | | | | | 1 Ch | 1 |
| *S. kentucky* | | | | | 1 | | 1 |
| *S. stanley* | | | | | | | 1 |
| *S. rubislaw* | 1 | | | | | | 1 |
| *S. worthington* | | | | | | | 1 |
| *S. saint paul* | 6 | | | | | | |
| *S. muenchen* | 1 | 1 | 2 P | 3 | | 1 | |
| *S. pullorum* | | | 12 Ch | | | | |
| *S. gallinarum* | | | 2 Ch | | | | |
| *S. wichita* | | | | 1 | | | |
| *S. marseille* | | | | 1 | | | |
| *S. rostock* | | | | | 1 | | |
| *S. gaminara* | | | | | | 1 P | |

Ch = chickens
P = pork
M = milk
C = cattle
B = beef
Pi = pig
R = rat

1) Felsenfeld *et al.*, 1953
2) Felsenfeld and Young 1948
3) Felsenfeld and Young 1949

In the case of animals which had been previously slaughtered or had died, smears were taken with one and the same swab from the mucosa of the following sections of the alimentary tract: pylorus, duodenum, ileum, ileocaecal valve, colon and rectum.

Normal bacteriological examination of the flesh was also carried out in the case of slaughter animals.

To trace *Salmonella* hosts among living animals, the so-called rectal swab method was employed, during which the throat swab referred to in the foregoing was inserted 10–12 cm into the rectum.

Esbach brilliant green bouillon was originally used as a nutrient medium, the culture being transferred to Endo- or S.S. slides after 12–16 hours incubation. Towards the middle of 1955, tetrathionate bouillon (Difco), combined with brilliantgreen-phenolred agarplates (Difco), was adopted instead, as a result of which the number of positive reactions showed a considerable increase; this may be explained by the greater selectivity of the latter combination and the simplified recognition of suspicious colonies.

One or more suspicious colonies were subsequently inoculated in tubes of bouillon agar. After sealing with paraffined corks these cultures were forwarded by sea or air-mail to the National Salmonella Centre of the National Institute of Public Health, Utrecht, The Netherlands, for further examination.

## 2. Examination of imported slaughter cattle (beef cattle and goats).

Until the end of 1953, Aruba obtained practically all its slaughter cattle from Colombia. During the last quarter of that year, however, confirmation of foot and mouth disease in a consignment compelled beef importers to make their subsequent purchases in another country. The choice fell initially on Costa Rica, where a good type of beef cattle is bred. The quality of the latter was such that it was assumed that – notwithstanding the longer voyage and all the attendant disadvantages and risks – importing this cattle would nevertheless constitute a paying proposition. Subsequent events, however, did not justify this optimism, as both during shipment as well as during the period following disembarkment fairly heavy losses occurred.

In 1954, for example, 25 carcasses out of a total shipment of 320 head had to be disposed of overboard during the voyage to the Netherlands Antilles, while a further 30 dead animals were found in the ship on its arrival at the port of destination. During their ensuing stay in the cattle pens of the slaughter houses, a further 15 of the remaining animals died, bringing the total losses to more than 20 %. The ship had experienced very rough weather, with the result that all the animals were completely exhausted and had suffered serious bruising during the voyage. It was thought that the deaths could be attributed to these factors, as also the symptoms found on arrival, *viz.* constipation and darkly coloured, fluid faeces. It became evident that this supposition was erroneous when numerous deaths were reported in the following consignment, which had been shipped under favourable conditions, coupled with the fact that the animals still alive on arrival evidenced similar symptoms to those shown by the previous shipment.

Post-mortem examinations revealed the following aberrations: swelling of the parenchymatous organs, generally without petechia; haemorrhagic oedema of the abomasum, occasionally attended by petechial haemorrhage; chronic ulcers were not infrequent, especially on the pylorus; haemorrhagic-fibrinous or purulent infection of the entire intestinal canal; marked haemorrhages in the mucous membrane of the ileocaecal valve.

An extensive, acute fibrinous peritonitis, initiated by ulcers in the abomasum, was also found in two oxen.

The diagnostic symptoms indicated Anthrax, Pasteurellosis and Salmonellosis as possibilities. Subsequent investigations yielded negative results in respect of the first two of these diseases, while *Salmonella* could be cultivated from the intestinal contents, as well as from the liver and gall bladder, in ten animals. Subsequent examinations indicated that the *Salmonella* types involved were *S. anatum* and *S. typhi murium.*

Consequent upon these findings, 340 head of beef and 150 goats, imported as slaughter cattle, were subjected to further examination during the course of 1954 and 1955. This elicited the fact that *Salmonella* infections were not restricted to cattle from Costa Rica, but also occurred in animals imported from other countries. It also appeared that the conditions prevailing during shipment and especially those under which the animals were kept in Aruba, were extremely important factors governing the outbreak of serious cases. The results of the bacteriological investigations are summarized in Table 2.

The animals, it will be noted, are classified in two groups, *viz.* those examined within 48 hours after their arrival in Aruba and those examined subsequently. This distinction was made for the purpose of differentiating between infections which could definitely be regarded as having been imported and those which might

TABLE 2.

*Salmonella* found in slaughter animals imported into Aruba 1954 and 1955.

| Country of origin | Animals examined | | No. positive | | After a stay of from 2 days to 3 weeks in Aruba | *Salmonella* | |
|---|---|---|---|---|---|---|---|
| | | | immediately or within 48 hrs after arrival | | | | |
| | animal | number | Rectal examination 1) | Post-slaughter examination | Post-slaughter examination | species | No. |
| Dominican Republic | beef | 115 | 14 (30) | | 48 | *S. anatum* | 23 |
| | | | | | | *S. havana* | 23 |
| | | | | | | *S. oranienburg* | 1 |
| | | | | | | *S. typhi murium* | 19 |
| Costa Rica | beef | 130 | 11 (64) | 4 | 60 | *S. anatum* | 12 |
| | | | | | | *S. havana* | 17 |
| | | | | | | *S. panama* | 18 |
| | | | | | | *S. eastbourne* | 2 |
| | | | | | | *S. typhi murium* | 9 |
| | | | | | | *S. saint paul* | 2 |
| | | | | | | *S. oslo* | 8 |
| Honduras | beef | 95 | 13 (90) | 6 | 25 | *S. anatum* | 16 |
| | | | | | | *S. give* | 4 |
| | | | | | | *S. newport* | 8 |
| | | | | | | *S. oranienburg* | 4 |
| | | | | | | *S. typhi murium* | 2 |
| | | | | | | *S. enteritidis* | 1 |
| | | | | | | *S. eastbourne* | 3 |
| | | | | | | *S. denver* | 1 |
| | | | | | | *S. arechavaleta* | 2 |
| | | | | | | *S. kisangani* | 1 |
| | | | | | | *S. glostrup* | 1 |
| | | | | | | *S. reading* | 1 |
| Colombia | goat | 150 | 2 (6) | 36 | | *S. glostrup* | 19 |
| | | | | | | *S. carrau* | 3 |
| | | | | | | *S. oranienburg* | 1 |

1) The total number of animals examined is indicated in parentheses.

be attributable to an infection occurring on the island itself.

As will be seen from the table, a larger number of *Salmonella* species were found among the group of animals examined later than 48 hours after their arrival. This is partially explained by the more detailed examination which took place in the case of slaughtered animals.

### 3. Examination of indigenous animals.

**a.** Smaller dairy animals (goats, sheep and pigs). It proved possible to extend the investigation to embrace a total of 179 animals. The results are collected in Table 3.

TABLE 3.

Examination of indigenous animals.

| Animal | No. of animals examined | Positive *Salmonella* reaction | Percentage positive reactions |
|---|---|---|---|
| Goat | 63 | 8 | 12 |
| Sheep | 11 | 2 | 18 |
| Pig | 105 | 16 | 15 |

In addition, a number of pig farms were visited for inspection purposes; our findings are incorporated, together with those in respect of slaughtered animals, in Table 4.

TABLE 4.

*Salmonella* species isolated from indigenous animals.

| *Salmonella* species | Goat | Sheep | Pig |
|---|---|---|---|
| *S. anatum* | + | | + |
| *S. carrau* | + | | + |
| *S. glostrup* | + | | + |
| *S. oranienburg* | + | + | + |
| *S. canoga* | | + | + |
| *S. muenster* | | + | |
| *S. arechavaleta* | | | + |
| *S. havana* | | | + |
| *S. manhattan* | | | + |
| *S. panama* | | | + |
| *S. saint paul* | | | + |
| *S. typhi murium* | | | + |
| *S. abaetetuba* | | | + |
| *S. newport* | | | + |

**b.** Dogs and cats.

Of 18 dogs and 15 cats, examined either alive or postmortem, 9 dogs and 7 cats were found to be infected with *Salmonella*.

A number of the animals examined were frequently fed on offal from the slaughter-house. As was also found in the case of indigenous pigs, practically all animals without exception fed on this offal were infected with *Salmonella*. The results of this investigation are summarized in Table 5.

**c.** Canaries.

A noteworthy fact is the peracute death of ca 80 canaries following a *S. typhi murium* infection.

TABLE 5.

*Salmonella* species isolated from indigenous dogs and cats.

| *Salmonella* species | dog | cat |
|---|---|---|
| *S. anatum* | + | + |
| *S. havana* | + | + |
| *S. glostrup* | + | |
| *S. oranienburg* | + | + |
| *S. manhattan* | + | |
| *S. panama* | + | |
| *S. typhi murium* | + | + |
| *S. arechavaleta* | | + |
| *S. derby* | | + |

A pure culture of this *Salmonella* could be obtained from the cardiac blood of a number of dead birds, while a collective sample of the faeces likewise showed a positive reaction.

Despite the therapy adopted [1]) and the subsequent recovery of clinically diseased birds, faeces samples examined 1 or 2 weeks later still contained *S. typhi murium.*

**d.** Rodents.

This investigation was restricted to the examination of the faeces of the white mice bred at the Government Stock Farm for the use of the Veterinary Service. Three *Salmonella* species were isolated, *viz., S. manhattan, S. typhi murium* and *S. braenderup.*

**e.** Summary of the results.

The findings in all the investigations referred to in the foregoing are summarized in Table 6. For comparative purposes, the types of *Salmonella* found in man in the Netherlands Antilles are also listed in this table, together with those found in animals.

## E. DISCUSSION.

To ensure adequate meat supplies Aruba is compelled to import slaughter cattle, with the result that *Salmonella* carriers are constantly being introduced into the island. This must be regarded as a potential danger to public health, a fact which had to be considered when carrying out veterinary cattle and meat inspections.

The economic aspect of the problem is far from negligible and it

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[1]) Chloramphenicol 100 mg/kg live weight and terramycine 15 mg/kg live weight in the drinking water for a period of 5 days.

TABLE 6.

*Salmonella* species found in man and animal in the Netherlands Antilles, especially Aruba.

| | | No. of cases in the Neth. Antilles 1933—1953 | | No. of cases in Aruba 1954—1955 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| group | *Salmonella* species | man | animal | slaughter animals | | | | | | | |
| B | *S. kisangani* | | | 1 | | | | | | | |
| | *S. arechavaleta* | | | 2 | | | 1 | | 1 | | |
| | *S. saint paul* | 7 | | 4 | | | 3 | | | | |
| | *S. reading* | | | 1 | | | | | | | |
| | *S. derby* | 3 | | | | | | | 1 | | |
| | *S. typhi murium* | 19 | 3 (beef) | 31 | | | 1 | 1 | 4 | 4 | 1 |
| C . 1 | *S. oslo* | | | 8 | | | | | | | |
| | *S. denver* | | | 1 | | | | | | | |
| | *S. braenderup* | | | | | | | | | | |
| | *S. montevideo* | 16 | | | | | | | | | |
| | *S. oranienburg* | 20 | | 5 | 2 | 1 | 4 | 2 | 2 | | |
| C. 2 | *S. muenchen* | 5 | | | | | | | | | |
| | *S. manhattan* | | | | | | 3 | 1 | | | 1 |
| | *S. newport* | 16 | | 8 | | | 1 | | | | |
| | *S. glostrup* | | | 1 | 22 | | 1 | 1 | | | |
| D | *S. eastbourne* | | | 5 | | | | | | | |
| | *S. enteritidis* | 27 | | 1 | | | | | | | |
| | *S. panama* | 5 | | 18 | | | 1 | 1 | | | |
| E | *S. muenster* | 2 | | | | 1 | | | | | |
| | *S. anatum* | 2 | | 63 | 2 | | 4 | 7 | 1 | | |
| | *S. give* | 3 | | 4 | | | 2 | | | | |
| | *S. canoga* | | | | | 1 | 1 | | | | |
| F | *S. abaetetuba* | | | | | | 2 | | | | |
| G | *S. havana* | | | 45 | | | 6 | | 1 | | |
| H | *S. carrau* | 1 | | | 4 | | 2 | | | | |
| | *S. thompson* | 1 | | | | | | | | | |
| | *S. typhi* | 55 | | | | | | | | | |
| | *S. bredeney* | 3 | | | | | | | | | |
| | *S. illinois* | 1 | | | | | | | | | |
| | *S. paratyphi B* | 29 | | | | | | | | | |
| | *S. cholerae suis* | 1 | 2 (pig) | | | | | | | | |
| | *S. rubislaw* | 1 | | | | | | | | | |

is therefore absolutely essential to curtail the attendant risks as far as possible by due care and attention, both during transit as well as after disembarkation. There appears to be every justification for the assumption that the costs involved will be more than offset by the higher returns.

In view of the fact that a very high percentage of the pigs fed on slaughter-house offal were found to be infected with *Salmonella*, the correlation between this infection and their fodder is obvious. With these considerations in mind, it is thus highly desirable that slaughter-house offal destined for use as pig fodder should be treated to ensure that all *Salmonella* germs therein are killed.

As *Salmonella* carriers are frequently found among dogs and cats in Aruba, this also being partially linked up with their feeding on slaughter-house offal, measures as sterilization of slaughter-house offal and the provision of effective fencing, etc., around the slaughter-house premises, should likewise be taken in respect of these animals.

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## Summary.

The authors describe an investigation of the occurrence of *Salmonella* among animals in Aruba in 1954/55, embracing both slaughter cattle (beef and goats) imported from various countries, as well as indigenous animals (goats, sheep, pigs, dogs, cats, canaries and mice). The results of these investigations are summarized in Table 6. Although only one subculture was examined as a rule, no less than 32 different *Salmonella* species were found.

## Résumé.

Les auteurs décrivent un examen de la présence du *Salmonella* dans les animaux de l'île d'Aruba en 1954/55. Les animaux étaient d'une part d'origine étrangère (des bovins et des chèvres) et d'autre part d'origine intérieure (des chèvres, des brebis, des porcs, des chiens, des chats, des canaris et des souris). Les résultats de cettes recherches sont résumés dans la table 6. Bien que le plus souvent seulement une subculture fût examinée, non moins que 32 différents types de *Salmonella* furent isolés.

## Zusammenfassung.

Die Verfasser beschreiben eine Untersuchung über das Vorkommen von *Salmonella* bei Tieren auf der Insel Aruba während der Jahre 1954/55. Es handelt sich hierbei sowohl um importierte Tiere (Rinder und Ziegen) aus verschiedenen Ländern, als um auf der Insel lebende Tiere (Ziegen, Schafe, Schweine, Hunde, Katzen, Kanarievögel und Mäuse). Die Ergebnisse dieser Untersuchungen sind in Tabelle 6 zusammengefasst. Obwohl meist nur eine Subkultur untersucht wurde, konnten doch nicht weniger als 32 *Salmonella*-Typen isoliert werden.

## Resumen.

Los autores describen una investigación referente a casos de *Salmonella* presenciados en animales de Aruba, durante 1954/55, en que fueron implicados tanto reses de matanza (incluyendo vacunos y cabras), cuya importanción procedía de varios países, como animales procedentes del suelo aborigen (cabras, carneros, cerdos, perros, gatos, canarios y ratones). Los resultados obtenidos a raíz de estas investigaciónes quedan resumidos en la Tabla No. 6. Aunque, por regla general, se examinó un solo subcultivo, no fueron encontrados nada menos que 32 tipos diferentes de *Salmonella*.

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# THE ENUMERATION OF PROTEOLYTIC BACTERIA IN FOODS

by

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In a recent extensive British survey the total number of viable bacteria per gramme of food ('total plate count') proved to be very helpful for the recognition of toxic foods (9, 10). It appeared that foods containing more than about $10^4$/gramme of viable bacteria are definitely not always harmful. However, they may very well be toxic, *viz.* if among the bacteria present some happen to be of types, which are toxinogenic – though not yet recognized as such, because they do not belong to the classical food poisoning genera, such as salmonelleae, staphylococci, clostridia, etc.

For the practice of the microbiological examination of foods, it seems appropriate to adopt the inverse of these results as a guiding principle, *i.e.* to consider as safe foods, those, which contain neither the classical food poisoning bacteria nor a total number of viable bacteria well over $10^4$/gramme. There is, however, an important exception to this general rule of rejecting foods with plate counts well over $10^4$, *viz.* when analyzing foods, regularly or sometimes obtained by microbial fermentation. Commodities of this type are sauerkraut and pickles; durable sausage (5, 21, 20); cream, butter, cottage cheese (13, 8), yoghurt and margarine (1). For obvious reasons the total plate count of such foods will often amount to many millions per gramme and so without leading to suspecting its bacteriological status, because the acidogenic bacteria concerned are well known never to be detrimental to human health. What is needed in this case to recognize potentially dangerous contaminants is a count of non-acidogenic, but rather proteolytic bacteria.

In Dairy Microbiology for this purpose mostly an indirect method *i.e.* a determination of the catalase-value of the product is applied. This technique seems not very suitable for three reasons, *viz.* (i) although most lactic acid bacteria are indeed catalase-negative, an important class of proteolytic food spoilage bacteria, *i.e.* the clostridia, is also catalase-negative and is therefore not accounted for; (ii) the catalase-technique does not permit the isolation of the catalase-positive strains for further study; (iii) the catalase-value of raw meat or cheese may seriously interfere with the enumeration of microbes.

The practice of basing a hygienic evaluation on the direct count of proteolytic bacteria has been used in Water Bacteriology for many decades (4), though its popularity seems to decrease (23, 24). For this purpose the water samples are seeded in melted peptone/meat extract/gelatin; after suitable incubation at 20–25° C. the gelatinolytic colonies are counted.

When tentatively applying this method to foods, subjected to a complete bacteriological analysis (18) in order to decide whether they could be considered wholesome, we had to conclude that it was not very well suited for our purpose. Already in general many inconveniences are presented by the use of gelatin plates, such as difficulty in handling them in warm weather and spoilage by liquefying organisms. Their special application to foods was further limited by the obligatory use of an incubation temperature below the growth range of some bacteria occurring in foods and suspected as potential causes of food poisoning.

It was therefore decided to use Frazier's gelatin/agar (6), currently applied in Determinative Bacteriology, instead. In this medium gelatinolysis is demonstrated by flooding the incubated plates with a 'developer', consisting of corrosive sublimate, dissolved in hydrochloric acid.

It appeared soon that this technique cannot be used for making pour plates. Hence we applied the surface plating principle, originally introduced by BURRI in 1928 (3) and successfully used since in many phases of food bacteriology (11, 22, 17, 14).

## PROCEDURE.

### Medium.

The medium used, was a modern variety of the composition, originally recommended by FRAZIER (6). It contains 5 g tryptone

(Difco), 3 g yeast extract powder (Difco), 4 g gelatin (Difco) and 15 g agar per liter distilled water. If necessary, the pH of the medium is brought to 7.0 with $K_2HPO_4$ solution. It is sterilized for 15 min. at 121° C.

Plates are poured with an attempted layer thickness of about 2 mm, corresponding to about 10 ml of agar per plate. After solidification the plates are dried for about 15 min. at 55° C.

### Inoculation and incubation.

From the food under investigation a set of serial decimal dilutions is prepared in such a way, that at least one dilution will yield between 5 and 50 colonies per 0.05 ml.

Inoculation of the plates is carried out with the help of bacteriological pipettes, graduated to 0.1 ml. Drops of 0.05 ml are deposited in the centre of the plates and thereupon spread out with the help of a sterile bended glass rod, a so-called Drigalski spatula.

Plates so inoculated are left at room temperature for five minutes and then incubated for 24–48 hours both at the classical temperature of 22 ± 1° C. and at 31 ± 1° C.; the latter being the range found in a previous investigation (19) to be the best single temperature for food spoilage bacteria.

### Development of the incubated plates.

After incubation first of all the total numbers of colonies present on the plates are counted.

Thereupon the plates are flooded with the 'developer' solution. This solution is prepared by dissolving 15 g of $HgCl_2$ in 20 ml of pure, 38% HCl, and subsequently diluting this solution with 100 ml distilled water. After a few minutes both the sublimate solution and the soaked off colonies are poured off and the plates washed once again with tap water.

On the 'developed' plates so obtained the numbers of halos are counted, every non-confluent halo representing one gelatinolytic colony. Plates should preferably show between 3 and 30 of such halos.

If gelatinolytic colonies are to be studied morphologically or physiologically, they must be harvested, prior to developing the plates, preferably with LEDERBERG's replica plating technique (12).

## RESULTS.

### Pure culture studies with aerobes and facultative anaerobes.

The technique was first of all applied in duplicate series of tests to twelve gelatinolytic species, *viz.*:

a) three strains of coagulase-positive *Staph. aureus*, obtained from Professor RUYS, Laboratory of Hygiene, University of Amsterdam (17);

b) one strain of *Klebsiella cloacae*, isolated from raw milk by GALESLOOT (7), and two strains of *Proteus vulgaris* from human faeces;

c) one strain of *Streptococcus liquefaciens*;

d) two strains of bacilli, *viz. B. subtilis* (Marburg strain) and *B. cereus* (Smith strain nr 232);

e) two strains of psychrophilic proteolytic bacteria, *viz.* an *Achromobacter* species, isolated from refrigerated meat by Dr M. INGRAM (Cambridge) and one *Pseudomonas* strain, isolated by ourselves from slimy, refrigerated fish (15).

f) one strain of *Candida lipolytica*, used in a previous investigation on microbial lipolytic spoilage of foods (16).

The results which were obtained are recorded in Table 1. It may be concluded, that the productivity of the technique is excellent. As was to be expected, the psychrophilic strains, though growing at 31° C. (19), did not liquefy gelatin at that temperature; while three mesophilic strains, although quantitatively recovered at 22°C., did not form halos at that temperature.

For the purpose of judging the wholesomeness of fermented foods, therefore, the temperature of 31° C. appeared again to be the best single one, just as was found in a previous investigation (19) on the optimal single temperature for carrying out total plate counts of foods.

### Use in the bacteriological analysis of indicated foods.

In view of the favourable results obtained with pure cultures it was decided to apply the method to a few foods of the type, for which it was developed, *i.e.* mature durable sausage; butter, cream and cottage cheese; and margarine.

Of each commodity at least six different samples of apparently good quality were analysed. For this purpose $10^{-1}$ and $10^{-2}$ dilutions in

### TABLE 1.

Standard poured plate count per ml of pure culture in tryptone/dextrose/yeast extract/agar (2) and surface plate count per ml of pure culture on Frazier-agar of twelve strains of food spoilage organisms.

| Strain | Plate count (2 days at 31° C.) | Frazier surface plate count (2 days at 31° C.) | | Plate count (2 days at 31° C.) | Frazier surface plate count (2 days at 22° C.) | |
|---|---|---|---|---|---|---|
| | | Number of colonies | Gelatinase *) | | Number of colonies | Gelatinase *) |
| *Staph. aureus* 1850 | $3.8 \times 10^6$ | $4.3 \times 10^6$ | + | $1.4 \times 10^7$ | $1.3 \times 10^7$ | + |
| *Staph. aureus* 1878 | $9.7 \times 10^6$ | $8.8 \times 10^6$ | +++ | $5.7 \times 10^6$ | $2.6 \times 10^6$ | ++ |
| *Staph. aureus* 1910 | $8.7 \times 10^6$ | $8.8 \times 10^6$ | +++ | $8.7 \times 10^6$ | $7.5 \times 10^6$ | ++ |
| *Klebsiella cloacae* | $1.4 \times 10^7$ | $1.5 \times 10^7$ | + | $2.7 \times 10^6$ | $3.5 \times 10^6$ | — |
| *Proteus*, strain B | $2.5 \times 10^6$ | $4.2 \times 10^6$ | +++ | $4.5 \times 10^7$ | $4.9 \times 10^7$ | +++ |
| *Proteus*, strain W | $1.6 \times 10^6$ | $1.1 \times 10^6$ | + | $4.7 \times 10^7$ | $5.5 \times 10^7$ | — |
| *Str. liquefaciens* | $1.6 \times 10^6$ | $2.1 \times 10^6$ | ++ | $1.6 \times 10^6$ | $1.5 \times 10^6$ | — |
| *B. subtilis* | $1.3 \times 10^7$ | $2.2 \times 10^7$ | +++ | $1.3 \times 10^7$ | $2.3 \times 10^7$ | ++ |
| *B. cereus* | $2.5 \times 10^4$ | $4.7 \times 10^4$ | ++++ | $1.6 \times 10^4$ | $3.5 \times 10^4$ | ++++ |
| *Pseudomonas*, strain K II | $6.2 \times 10^6$ | $5.1 \times 10^6$ | — | $1.9 \times 10^7$ | $2.1 \times 10^7$ | ++ |
| *Achromobacter*, strain E | $1.3 \times 10^7$ | $1.3 \times 10^7$ | — | $1.3 \times 10^7$ | $1.3 \times 10^7$ | + |
| *Cand. lipolytica* | $5.6 \times 10^5$ | $7.1 \times 10^5$ | ++ | $1.7 \times 10^6$ | $2.8 \times 10^6$ | ++ |

*) Legend: — = no gelatinolytic colonies; + = all colonies gelatinolytic, diameter about 1 mm; ++, etc. = all colonies gelatinolytic with diameters resp. 2 to over 10 mm.

saline, resp. in the ease of butter and margarine the serum obtained at 43 ± 1°C., were streaked in quantities of 0.05 ml on Frazier gelatin plates and developed after 2 days incubation at 31 ± 1° C. The results obtained are reported in Table 2.

TABLE 2.

Total plate counts and numbers of gelatinolytic bacteria in some current foods.

| Food | Total plate count/g | Gelatinolytic organisms/g |
|---|---|---|
| **Butter** *) | | |
| Sample nr 1 | $8.0 \times 10^2$ | $5.0 \times 10^2$ |
| 2 | $> 10^6$ | $3.4 \times 10^3$ |
| 3 | $9.0 \times 10^4$ | $1.2 \times 10^3$ |
| 4 | $1.0 \times 10^4$ | $2.6 \times 10^2$ |
| 5 | $> 10^6$ | $< 20$ |
| 6 | $1.6 \times 10^5$ | $1.4 \times 10^4$ |
| 7 | $> 10^6$ | $3.8 \times 10^3$ |
| 8 | $5.0 \times 10^3$ | $3.2 \times 10^2$ |
| 9 | $> 10^6$ | $4.0 \times 10^3$ |
| 10 | $9.0 \times 10^3$ | $2.6 \times 10^3$ |
| **Cottage and cream cheese** | | |
| Sample nr 1 | $1.3 \times 10^4$ | $7.8 \times 10^3$ |
| 2 | $8.8 \times 10^3$ | $6.4 \times 10^3$ |
| 3 | $6.0 \times 10^2$ | $6.0 \times 10^2$ |
| 4C | $2.1 \times 10^6$ | $6.0 \times 10^2$ |
| 5C | $5.1 \times 10^5$ | $5.1 \times 10^3$ |
| 6C | $2.3 \times 10^5$ | $4.2 \times 10^3$ |
| **Cream** | | |
| Sample nr 1 | $6.0 \times 10^2$ | $< 200$ |
| 2 | $4.0 \times 10^2$ | $2.0 \times 10^2$ |
| 3 | $5.6 \times 10^3$ | $4.0 \times 10^2$ |
| 4 | $6.0 \times 10^3$ | $< 200$ |
| 5 | $> 10^6$ | $> 10^5$ |
| 6 | $2.4 \times 10^4$ | $1.8 \times 10^4$ |
| 7 | $1.2 \times 10^7$ | $8.0 \times 10^5$ |
| 8 | $4.0 \times 10^2$ | $2.0 \times 10^2$ |
| 9 | $2.0 \times 10^6$ | $4.8 \times 10^4$ |
| 10 | $1.1 \times 10^5$ | $2.6 \times 10^4$ |
| **Durable sausage** | | |
| Sample nr 1 | $> 10^7$ | $< 200$ |
| 2 | $> 10^7$ | $8.0 \times 10^2$ |
| 3 | $> 10^7$ | $3.8 \times 10^4$ |
| 4 | $> 10^7$ | $< 200$ |
| 5 | $> 10^7$ | $6.0 \times 10^2$ |
| 6 | $> 10^7$ | $6.8 \times 10^3$ |
| 7 | $> 10^7$ | $< 200$ |
| 8 | $> 10^7$ | $< 200$ |
| 9 | $> 10^7$ | $< 200$ |
| 10 | $> 10^7$ | $< 200$ |
| **Margarine** *) | | |
| Sample nr 1 | $3.3 \times 10^7$ | $5.0 \times 10^2$ |
| 2 | $7.0 \times 10^7$ | $7.4 \times 10^2$ |
| 3 | $0.6 \times 10^2$ | $< 20$ |
| 4 | $5.8 \times 10^3$ | $3.0 \times 10^3$ |
| 5 | $3.6 \times 10^2$ | $0.8 \times 10^2$ |
| 6 | $6.2 \times 10^4$ | $2.6 \times 10^2$ |
| 7 | $1.5 \times 10^5$ | $1.8 \times 10^2$ |
| 8 | $0.6 \times 10^2$ | $< 20$ |

*) All counts based on the serum, rendered at 42 — 44° C.

The only samples, showing a count of proteolytic bacteria well over $10^4$ per gramme, *i.e.* two samples of cream, appeared later to have been manufactured in a plant, which had recently been criticized by the Food Inspection Board for a lack of adequate sanitation. Therefore it seems a liberal standard to require that the numbers of gelatinolytic bacteria in foods of this type shall not be over $10^4$ per gramme.

## Summary.

For the enumeration of proteolytic bacteria in foods, regularly or sometimes obtained by microbial fermentation, a surface plating technique, using Frazier's gelatin/agar, incubated for two days at 31 ± 1° C. and thereupon developed with sublimate solution, appeared useful.

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# IN MEMORIAM PROFESSOR DR JAN SMIT

## 1885—1957

On the 14th of June 1957 the Netherlands Society of Microbiology had to note the loss of one of its most prominent members. Professor Dr JAN SMIT had a never slackening interest in our society. He acted as its president from November 1938 to the end of 1949. In this period the reorganisation of our journal became a fact. It was largely due to his thoroughness that this reorganisation proved successful. Up till 1938 "Antonie van Leeuwenhoek" had appeared exclusively in the Netherlands language. Soon after SMIT was appointed a president the 6th volume of the Journal was issued in its new international shape and notwithstanding the adversity of the war it did not last long before it found its readers and contributors spread all over the world.

During his presidency times were hard for SMIT, for his laboratory and for the Netherlands Society of Microbiology. In 1942 SMIT was discharged by the German authorities. When he came back to Wageningen after the war, he found his house burnt down; his books and scientific papers had gone lost; his laboratory was in disorder and it seemed hopeless to restore it. However, after some years of hard labour scientific work could be taken up again. And in 1949 the president of the society succeeding him met with a normally functioning organization.

On the 6th of October 1956 many of our fellow members were present when SMIT took his leave as a director of the Laboratory for Microbiology at Wageningen. The period wherein he enjoyed his pensionable age was but short. In the spring of 1957 SMIT fell ill and though it seemed that he had recovered from a cardiac affection, he had to undergo an operation of the gall bladder, which caused the failure of his weak heart.

It is not our task to give a description of his scientific carrier in full detail, which has already been described in Chemisch Weekblad **52**, 729 – 733, 1956.

For his personal qualities JAN SMIT will be remembered by many bacteriologists, in his own country as well as abroad. He was a splendid speaker, which will be missed in the meetings of the Netherlands Society of Microbiology.

(Department of Microbiology, University of Oklahoma School of Medicine,
Oklahoma City 4, Oklahoma, U.S.A.).

# GROWTH INHIBITION OF *MICROCOCCUS PYOGENES* BY MANGANESE AND RIBOFLAVIN [1]

by

**H. H. RAMSEY** and **T. E. WILSON**

(Received June 6, 1957).

## INTRODUCTION.

During the course of screening experiments designed to test the ability of vitamins and other chemically defined biological materials to antagonize chloramphenicol, an unexpected growth inhibitory property of riboflavin was noted. Riboflavin has been shown to retard microbial growth when used at levels of 65 $\mu$g per ml or higher (FOSTER and PITTILLO, 1953), however the vitamin is usually considered to be non-toxic even at concentrations far above the usual physiological levels (WAGNER-JAUREGG, 1954). In view of this a limited investigation into the nature of the riboflavin inhibition was undertaken. It has been found that the vitamin is toxic at concentrations of 5 $\mu$g or less, provided manganese is present; iron has a limited capacity to reverse this inhibition.

## EXPERIMENTAL.

Two strains of *Micrococcus pyogenes* var. *aureus* were used. One of these (strain S) was a typical coagulase positive micrococcus, inhibited by 8 $\mu$g of chloramphenicol (CM) per ml. The second strain (strain R) had been derived from the first by serial transfer in increasing concentrations of CM. The results obtained with the two strains were comparable and therefore, for simplicity, only the results obtained with strain R are presented. Cultures of strain

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[1]) Supported, in part, by a research grant (E-605) from the National Institutes of Health, U.S. Public Health Service. A preliminary report has appeared (RAMSEY and WILSON, 1956).

R were maintained on slopes of AC agar (Difco) containing 500 μg of CM per ml. Inocula were prepared from 20–24 hour AC broth cultures which were washed twice in sterile saline.

A semi-synthetic medium, previously described (RAMSEY and PADRON, 1954), was used and modified by the addition or omission of material as desired for a particular experiment. Determination of vitamin requirements in the presence of CM was accomplished by omitting single vitamins from the complete synthetic medium which contained graded concentrations of the antibiotic. Due to the variable lag time and growth rate in the presence of different concentrations of CM the results were recorded as the presence or absence of visible growth after 128 hours incubation at 37° C.

Degradation of riboflavin was achieved by adjusting aliquots containing 10 μg per ml to pH values of 5.0 or 9.0 and irradiating them for a period of one hour with a Mineralite U. V. lamp (model R51) placed at a distance of 8 cm from the surface of the solution. The depth of the solution was 1.1 cm and the solutions were continuously agitated by a magnetic stirrer during the period of irradiation. Residual riboflavin was determined by the growth response of *Lactobacillus casei* ATCC 7469 to varying concentrations of the irradiated solutions added to Riboflavin Assay Medium (Difco). An untreated aliquot was used as the standard riboflavin solution.

Turbidimetric determinations were made at 525 mμ with a Bausch and Lomb Spectronic 20 Colorimeter.

The plating technique consisted of pipetting 0.05 ml of the diluted cell suspenstion onto the solidified agar and spreading it over the entire surface with a sterile bent glass rod. Anaerobic conditions were obtained by the use of alkaline pyrogallol in a desiccator jar from which the air was evacuated and partially replaced with $CO_2$ to a final concentration of 10%.

## RESULTS.

As indicated previously, the initial observation of riboflavin toxicity occurred as a result of screening various compounds for their ability to antagonize chloramphenicol. The results of a typical experiment are shown in Table 1.

The omission of all vitamins from the medium did not allow growth even in the absence of the antibiotic. The omission of niacin, or thiamin, for which partial requirements had been previously

TABLE 1.

Effect of vitamins on sensitivity of *M. pyogenes* (strain R) to chloramphenicol.

| Vitamin omitted | Highest concentration of CM allowing growth at 128 hrs. |
|---|---|
| None | 200 μg/ml |
| All | 0 |
| Thiamin | 100 |
| Niacin | 100 |
| Riboflavin | 500 |
| Ca pantothenate | 300 |
| Pyridoxamine | 250 |
| Inositol | 250 |
| p-aminobenzoic acid | 200 |
| Biotin | 200 |
| Folic acid | 200 |
| Cyanocobalamin | 150 |
| All except thiamin, niacin and riboflavin | 300 |
| All except thiamin, niacin | 400 |

demonstrated in the absence of the antibiotic (RAMSEY and PADRON, 1954), allowed scanty growth only in the lower concentrations of CM. In the absence of calcium pantothenate, pyridoxamine and inositol growth occurred in slightly higher concentration of antibiotic than in the control containing all vitamins. Omission of cyanocobalamine, folic acid, para-aminobenzoic acid and biotin had a negligible effect on the level of CM in which growth occurred. The omission of riboflavin from the medium allowed rapid growth in 500 μg of CM as compared to growth in only 200 μg of CM per ml in the presence of riboflavin. It is unlikely that this inhibition, resulting from the incorporation of riboflavin into the medium was due to antagonism of other vitamins or to the destruction of essential nutrients (WAGNER-JAUREGG, 1954). In a medium containing only niacin and thiamin growth occurred in the presence of 400 μg CM per ml (Table 1). However, when riboflavin was added, growth was inhibited by 300 μg of CM per ml. When riboflavin solutions were sterilized by filtration and added to the medium (after sterilization) the same effect was observed. Therefore it was concluded that inhibition was not due to destruction or binding of nutrients by riboflavin, but rather that riboflavin exerts a direct inhibitory effect on the organism.

Partial degradation of riboflavin by ultra-violet light was accompanied by a reduction in inhibitory activity. For example, the sample irradiated at pH 9.0, which contained lumiflavin as the main degradation product (WAGNER-JAUREGG, 1954), had lost its inhibitory effect; growth was identical to the control which contained no riboflavin. Furthermore, microbiological assay indicated that this treatment resulted in approximately 65% loss of riboflavin activity (Fig. 1).

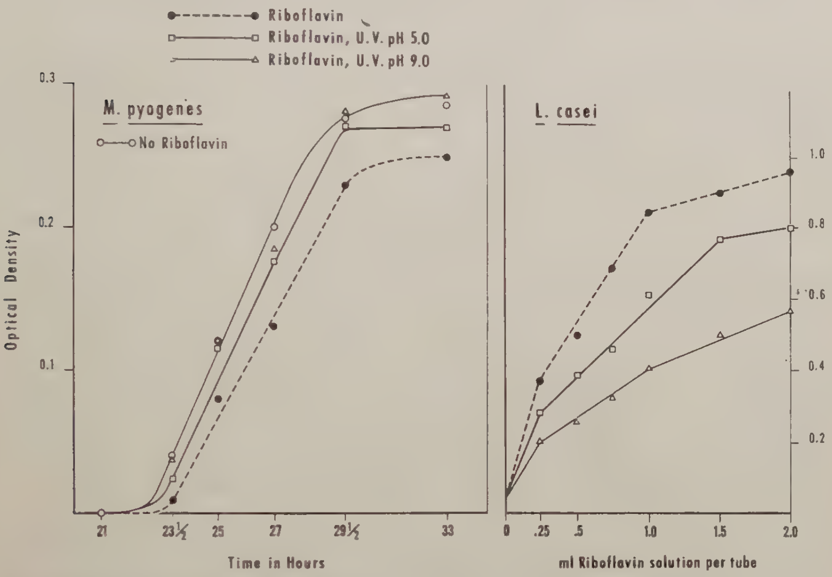

Fig. 1. Reduction of the inhibitory action of riboflavin by ultra-violet irradiation. Aliquots tested with *L. casei* diluted to equivalent of 0.1 μg/ml. Aliquots tested with *M. pyogenes* at a concentration of 2 μg/ml.

Partial destruction of riboflavin and a corresponding partial reduction of toxic activity resulted from irradiation at a pH of 5.0. It would seem that under these conditions riboflavin is the agent responsible for the prolongation of lag and alteration of the molecule results in decreased activity.

Further evidence for the direct participation of riboflavin was provided by chromatographic assay of riboflavin obtained from four commercial sources *). Although each sample exerted some toxic effect, the greatest inhibition was observed when the most chromatographically pure substance (Sigma Chem. Co.) was tested for activity. Thus, it appears that riboflavin *per se* or a very closely related compound (since riboflavin-5-phosphate was equal to riboflavin in inhibitory activity) is the inhibitory agent.

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*) Sigma Chemical Co., Fisher Scientific Co., California Foundation for Biochemical Research, Nutritional Biochemicals Co.

The active chelating properties of riboflavin have been described by ALBERT (1956) who noted an unusual avidity of riboflavin for ferrous iron. The removal or reduction of the level of certain trace elements in the medium would be sufficient to explain the inhibitory action of riboflavin. In order to examine this possibility, a two-fold increase in concentration of the various metals normally included in the medium were tested for their ability to reverse riboflavin inhibition. (Table 2).

TABLE 2.

The effect of increased concentration of metals on growth inhibition by riboflavin.

| Riboflavin conc. | Micrograms per ml [1] | | | | |
|---|---|---|---|---|---|
| | None | $K^+$ 1,796.0 | $Mn^{++}$ 6.5 | $Mg^{++}$ 78.4 | $Fe^{++}$ 4.0 |
| None | .30 [2] | .28 | .30 | .30 | .30 |
| 2 μg/ml | .18 | .20 | .04 | .18 | .30 |

[1] Two fold normal concentration.
[2] Optical density at 30 hours.

In the control series without riboflavin growth was quite uniform in all tubes. In the presence of 2 μg of riboflavin per ml growth was slower in all tubes except those containing a two-fold concentration of ferrous iron (4.0 μg/ml); in this instance growth was equal to that obtained in the control tubes without riboflavin. A twofold concentration of manganese (6.5 μg/ml) caused a striking inhibition of growth in the presence of riboflavin. Furthermore, increasing concentrations of ferrous iron did not proportionally reverse the inhibition caused by higher concentrations of either manganese or riboflavin. Ferrous iron was more effective in antagonizing the inhibition resulting from increased manganese concentration than that which resulted from high riboflavin concentrations. Cobalt and molybdenum were without effect.

Increasing concentrations of riboflavin were progressively effective in inhibiting growth initiation although there was little alteration in the amount of total growth attained (Fig. 2). Manganese also exerted a toxic effect when present in sufficiently high concentrations. The inhibitory action of either manganese or riboflavin was greatly enhanced by an increased concentration of the other.

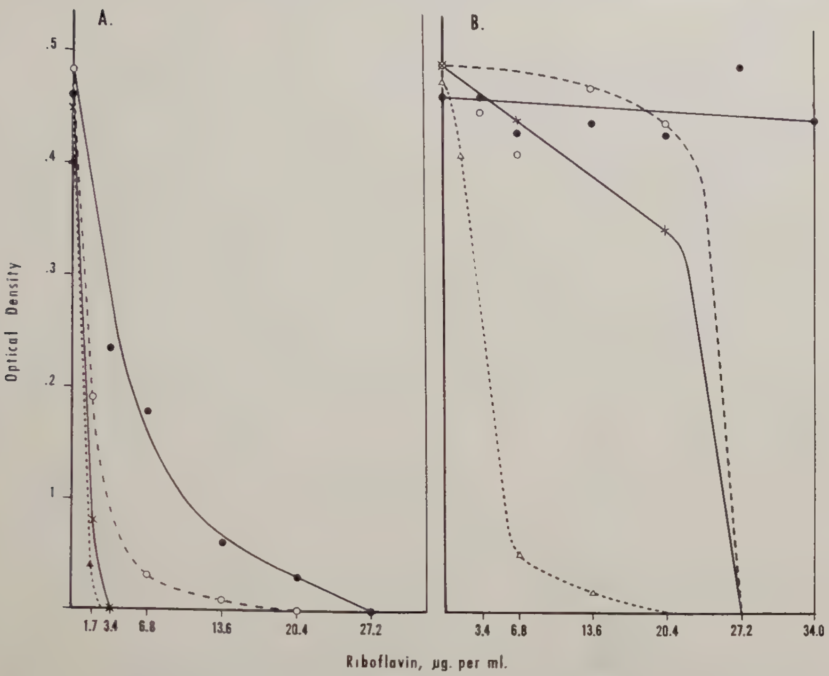

Fig. 2. The effect of increasing concentrations of riboflavin and manganese upon growth initiation. $FeSO_4$ omitted from the medium. A, 29 hours incubation; B, 44 hours incubation.

●—● No $Mn^{++}$ ×—× 1.0 µg $Mn^{++}$ per ml
○—○ 0.5 µg $Mn^{++}$ per ml △—△ 2.0 µg $Mn^{++}$ per ml

TABLE 3.

Antagonism of riboflavin-manganese inhibition by anaerobiosis.

| Condition of incubation | Riboflavin µg/ml | Micrograms of manganese per ml | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 |
| Aerobic | 0 | 56 1) | 51 | 60 | 55 | 46 |
| | 5 | 50 | 19 | 0 | 0 | 0 |
| Anaerobic | 0 | 59 | 54 | 60 | 49 | 46 |
| | 5 | 40 | 26 | 15 | 23 | 27 |

1) Average colony count at 72 hours

The dependency of the riboflavin-manganese inhibitory effect upon aerobiosis was demonstrated in a plating experiment (Table 3). Under both anaerobic and aerobic conditions only slight variations were observed in the number of colonies developing on the plates which did not contain riboflavin. Under aerobic conditions riboflavin and manganese combinations were quite inhibitory; the presence of 5 μg of riboflavin and 2 μg of manganese per ml prevented the development of visible colonies during the period of observation. Riboflavin, without added manganese, caused some reduction of the number of colonies which developed anaerobically. On all plates which contained both riboflavin and manganese anaerobiosis caused a marked negation of the inhibitory effect. For example, 45.7 % of the number of colonies appearing on the anaerobic control plate with neither riboflavin or manganese, developed on the plates containing 4 μg of manganese and 5 μg of riboflavin per ml. This combination was completely inhibitory under aerobic conditions.

## Discussion.

The marked inhibition of growth which occurs when both riboflavin and manganese are present in the medium would indicate that this inhibition is the result of the formation of an inhibitory riboflavin-manganese complex rather than an additive effect of the two agents. The inhibition caused by high concentrations of riboflavin in the absence of added manganese could be due to contaminating traces of manganese in the various ingredients of the medium since no effort was made to purify the various constituents. In like manner, since the test organism was capable of synthesizing riboflavin it is possible that at least part of the observed toxicity of manganese could be due to the effect of the combination of manganese with endogenously produced riboflavin. The active chelating properties of riboflavin would support the idea that a complex which interferes with formation or function of an essential metallo-enzyme was responsible for toxicity. It might be expected that an increased concentration of the normal metal constituent of the enzyme system would counteract the inhibitory effect of manganese, at least to some extent. This was found to be the case with ferrous iron, although the antagonism was not stoichiometric and, above certain levels of manganese no reversal by iron was apparent. The high avidity of the riboflavin molecule for ferrous iron as compared to manganese would indicate that inhibition was more complex than simple competition of metal ions for a site of attachment on the riboflavin molecule.

Examples of toxic chelate complexes have been demonstrated by

Rubbo *et al.* (1950) and Albert *et al.* (1956). The overall picture of the toxic action of 8-hydroxyquinoline (oxine), and 2-mercaptopyridine N-oxide, as furnished by their results is that an iron or copper complex acts as a catalyst which promotes the destructive oxidation of a group (e.g., an SH group )on a metabolite or enzyme which is vital to the cells. This toxic action can be prevented by the addition of cobalt to the medium; probably replacing cobalt (removed by the chelating agent) which normally protects the group. This concept is supported by the fact that the toxic action can also be prevented by lowering the oxidation-reduction potential of the medium thus preventing the destructive oxidation, or bringing into play a new metabolic pathway which circumvents it. Such an alternate route has been demonstrated by Lenhoff *et al.*, 1956 in *Pseudomonas fluorescens*; at high oxygen tensions, the flavoprotein content, as measured by diaphorase activity, increased, while at lower oxygen tensions the cytochrome content of the cells was increased. The formation of an increased amount of cytochrome was also found to be dependent upon the presence of iron. It is possible that the ability of iron to antagonize the riboflavin-manganese inhibition of *Micrococcus pyogenes* is due to the increased formation of cytochromes which afford an alternate electron route rather than to a competition between the metal ions for a site in a flavin enzyme system.

## Summary.

Riboflavin, in the presence of manganese, inhibited the growth of *Micrococcus pyogenes* var. *aureus*. The inhibitory action was shown to be dependent upon aerobiosis, and was characterized predominately as a prolongation of the lag phase. Increasing concentrations of either manganese or riboflavin, in the presence of a constant quantity of the other, enhanced the inhibitory effect. Ultra-violet irradiation of riboflavin reduced its toxic capacity in proportion to the amount of riboflavin degraded. Riboflavin-5-phosphate was equally as inhibitory as riboflavin. Ferrous iron was capable of a non-stoichiometric antagonism at low concentrations of riboflavin and/or manganese.

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(Research Department, British Vinegars Ltd., Frome, Somerset, England).

# THE MECHANISM OF LOSS OF STARCH-PRODUCTION BY *ACETOBACTER PASTEURIANUM*

by

**J. L. SHIMWELL**

(Received July 22, 1957).

Two species in the genus *Acetobacter* are noteworthy for their production of polysaccharides; *A. xylinum* (Brown) Beijerinck characteristically produces cellulose, whilst *A. pasteurianum* (Hansen) Beijerinck produces starch (or at any rate a starch-like substance giving the blue-black colour with iodine). These are the reactions on which these two *Acetobacter* species have been established.

The production of starch by *A. pasteurianum*, however, has long been known to be an unstable characteristic, and it is notorious that cultures of this species, after laboratory maintenance for some time, often, (and, indeed, usually) lose their ability to produce starch. For this reason FRATEUR (1950) considers that starch production is too unreliable a criterion on which to base species differentiation. Accordingly he regards *A. pasteurianum* as a starch-positive variety of *A. rancens*, for when starch production has been lost, cultures of *A. pasteurianum* are indistinguishable from those of *A. rancens*.

Several workers, notably HENNEBERG (1926) and FRATEUR (1950) have also found, however, that cultures of *A. pasteurianum*, which, during laboratory cultivation, have lost the ability to produce starch, can be restored to their former activity in this respect by prolonged cultivation on some different medium. Thus FRATEUR states that such starchless cultures of *A. pasteurianum*, when grown on yeast extract fructose chalk media, regained starch production after a period of two months. Similarly HENNEBERG records that starch-producing power could be restored by growing the starchless cultures on beer media.

The production of cellulose by *A. xylinum*, however, has, in contrast, until very recently, withstood the test of time, *A. xylinum* having been regarded as one of the most firmly established and most easily recognisable of all *Acetobacter* species.

In 1954, however, SCHRAMM and HESTRIN showed that *A. xylinum* can give rise to non-cellulosic mutants, thus shaking the essential diagnosis of this species. I have shown elsewhere (SHIMWELL, 1956) that such celluloseless mutants of *A. xylinum* are indistinguishable from the species *A. mesoxydans* Frateur, and that the mutation is apparently reversible, cellulosic cultures indistinguishable from *A. xylinum* being obtained from non-cellulosic cultures identifiable as *A. mesoxydans*. Similarly *A. mesoxydans* can give rise to further mutants which have lost their ketogenic power, and then correspond to FRATEUR's diagnosis of the species *A. rancens*.

Following this a study of authentic representative strains of all FRATEUR's species has shown that in almost every case mutants have been already present in the mother cultures as received, and that these mutants correspond to other well known *Acetobacter* species. The following mutations have actually been encountered in this laboratory (SHIMWELL, 1957a and b):

(1) Loss of ability to produce cellulose (*A. xylinum* to *A. mesoxydans*).
(2) Gain of ability to produce cellulose (*A. mesoxydans* to *A. xylinum*).
(3) Loss of ketogenic power towards glycerol (*A. mesoxydans* to *A. rancens*).
(4) Loss of catalase (or at any rate the discovery of a catalase-negative strain of *A. xylinum* var. *maltovorans*).
(5) Gain of ability to ferment glucose (*A. ascendans* to *A. rancens*).
(6) Loss of ability to use ammonium salts as sole source of nitrogen (*A. mesoxydans*: no change of species, SHIMWELL, 1957 b).

In some cases the above biochemical mutations have been associated with change of colony form and/or type of growth on solid and/or liquid media. In others they have been independant of cultural characteristics.

In view of the foregoing it was no longer possible to regard the criterion of cellulose-production as inviolable. On the contrary it seemed to be as unstable and mutable as that of starch-production by *A. pasteurianum*. It therefore seemed likely that the gradual loss of starch-production by a culture of *A. pasteurianum* was not due to a gradual enfeeblement of starch-producing power on the part of all the cells in the culture, but to a sudden discontinuous change of certain cells whereby starchless mutants were produced, these overgrowing the starch-positive cells in certain media, so that in course of time starch-production appeared to have waned completely.

## EXPERIMENTAL.

It was found difficult to obtain a starch-positive strain of *A. pasteurianum*. Strains obtained from various sources, including those in the National Collection of Industrial Bacteria (N.C.I.B.) all proved to be starchless, as were also *Acetobacter acidum-mucosum* N.C.I.B. 8133 (subsequently regarded by T. K. WALKER – private communication – as a strain of *A. pasteurianum*, and strongly starch-positive when first isolated) and *A. kuetzingianum* N.C.I.B. 3924 (regarded by FRATEUR as a sub-variety of *A. pasteurianum*).

Through the intermediary of Prof. FRATEUR, however, a culture of *A. pasteurianum* was kindly supplied by Dr LA RIVIÈRE from the Delft collection. This proved to be strongly starch-positive, giving an instantaneous blue-black reaction when a little of the growth was covered with iodine solution.

This culture was therefore plated out by smearing on the surface of unhopped-beer agar plates in serial dilution. The majority of the colonies produced were of smooth convex, circular, opaque type, but a few translucent rough colonies were also found on several of the plates (see Fig. 1). Upon picking off these different colonies and transferring separately to agar slopes of the same media it was found that the smooth circular colonies gave rise to starch-positive growths, whilst the rough colonies were starch-negative. Similarly, on flooding the plates with iodine solution the circular colonies stained blue-black, whilst the rough ones stained brownish-yellow.

Cultures of these starch-positive and starch-negative strains were cultivated at 27° C. on agar slopes of the same medium for two months, being transferred to new media daily. Upon plating out such cultures at intervals the starch-positive cultures continued to give rise to a proportion of starchless colonies, whilst the starchless cultures did not give rise to starch-positive or to other colony types. It thus appeared that there was a mutation involving loss of starch-production, and that this was an irreversible one. All other things being equal, therefore, there would be, in such cultures, a "mutation pressure" in the direction of loss of starch production, which in

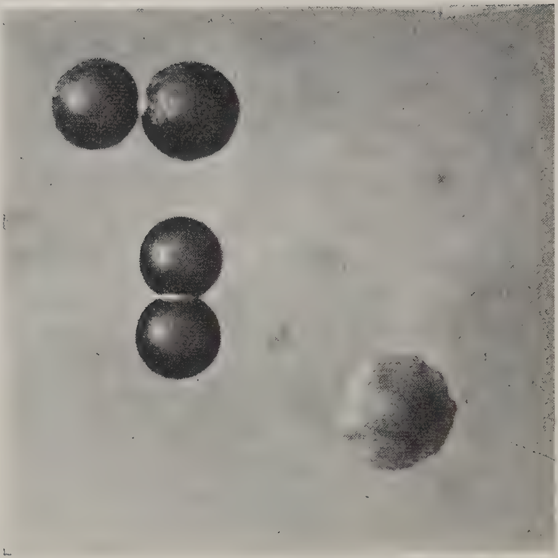

Fig. 1. Starch-positive (smooth) and starch-negative (rough) colonies of *A. pasteurianum* culture. × 40. Oblique transmitted light.

course of time would result in the gradual replacement of starch-positive by starch-negative cells. In the course of frequent transfer of, say, a relatively small portion of such culture to a larger quantity of fresh medium the number of starch-positive cells would tend to diminish, until eventually the inoculum would not contain any starch-positive cells at all, these having been diluted to extinction.

When once this point had been reached the culture would become an irreversibly starchless one, and attempts to restore the starch production by FRATEUR's (or any other) technique would no longer be successful. It is suggested that this is what has happened in the case of N.C.I.B. *A. pasteurianum* strains referred to above, whereas in the case of FRATEUR's restoration experiments a much shorter starch-losing period was allowed before the restoration process was commenced, there still being a few starch-forming cells present in the apparently starch-less inoculum.

## LOSS OF STARCH-PRODUCTION DUE TO CHANGE OF PHENOTYPE.

HENNEBERG (1926) states that a starch-positive culture of *A. pasteurianum* lost its starch-producing power after long cultivation on wort gelatin, but that the blue staining reaction returned in a "short time" when the culture was transferred to beer medium. This apparently rapid return of starch-production seemed to suggest a change of phenotype rather than of genotype.

Accordingly the starch-positive and starch-negative strains, isolated from the culture as described above, were cultivated on a variety of different solid media. It was at once found that the starchy cultures on unhopped-beer agar lost their starch-producing power at the very first transfer to malt wort agar, and immediately regained it on the very first transfer back to the original medium. Here, therefore, seemed clearly a case of change of phenotype.

On the other hand the starchless mutant would not produce any signs of starch on any of the media tried, as will be seen from the table below:

Starch Production.

| Medium | Starch-positive strain | Starch-negative mutant |
|---|---|---|
| Unhopped-beer agar | + | — |
| Yeast-extract calcium lactate agar | — | — |
| Yeast-extract glycerol agar | + | — |
| Malt-wort agar | — | — |

## Summary.

Experiments with an authentic, typical, starch-positive culture of *A. pasteurianum* have shown that the well known propensity of this species to lose the power of starch-production, (thereby becoming indistinguishable from *A. rancens*) may occur in two different ways:–

a. By an irreversible mutation involving a change of genotype, whereby mutants incapable of starch-formation are produced. The irreversible nature of the mutation establishes a "mutation pressure" tending, upon prolonged cultivation in certain media, to the eventual replacement of starch-positive by starch-negative cells.

b. By an immediate change of phenotype when the culture is transferred to media on which inherently starch-positive cells are unable to fabricate starch. This occurs at the very first transfer, and starch production is similarly restored immediately upon transfer back to the original medium.

A culture of the above mentioned starch-producing strain, together with the starchless mutant obtained from it, have been deposited with the N.C.I.B., Chemical Research Laboratory, Teddington, Middlesex, where they have been immediately freeze-dried.

## Acknowledgments.

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(Laboratory of Microbiology, Amsterdam, Holland and Institute „Jaime Ferrán" of Microbiology, Madrid, Spain).

# STUDIES ON BACTERIAL ESTERASES

## PART I. — DIFFERENTIATION OF A LIPASE AND TWO ALI-ESTERASES DURING THE GROWTH OF *PSEUDOMONAS AERUGINOSA* AND SOME OBSERVATIONS ON GROWTH AND ESTERASE INHIBITION [1]

by

**GONZALO SIERRA** [2]

(Received July 4, 1957).

### 1. INTRODUCTION.

The present paper deals with lipases and ali-esterases. The available information concerning esterases has mainly come from the study of animal and plant tissues. Although esterases, as a group, are specific, that is, that they are only capable to hydrolyse ester linkages, they apparently show among themselves a very low degree of specificity (ANDERSON, 1948). So any lipase will hydrolyse almost any ester, but there is a certain degree of specificity mainly in regard to the length of the chain of the acid (RAVIN and SELIGMAN, 1953; NACHLAS and SELIGMAN, 1949; HUGGINS and MOULTON, 1949; HUGGINS and LAPIDES, 1947; BALLS, MATLACK and TUCKER, 1937 and KASTLE, 1906).

At present, however, there is sufficient information to differentiate three groups which show some specificity. They are: 1. Lipase, which hydrolyses esters of longer chain fatty acids including fats and oils (NACHLAS and SELIGMAN, 1949). 2. Ali-esterases, which hydrolyse simple aliphatic esters of the lower fatty acids including triglycerides such as tributyrin and triacetin (RICHTER and CROFT,

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[1] This work was carried out in the Laboratory of Microbiology, Municipal University of Amsterdam, Holland, during the tenure of a grant from the "Consejo Superior de Investigaciones Cientificas', Spain and finished up during an assistantship at the Laboratory of Microbiology.

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1942). 3. Arom-esterases, which hydrolyse simple aromatic esters of the lower fatty acids and as unique characteristic hydrolyse paraoxon (diethyl p-nitrophenyl phosphate) and DFP (di-isopropyl fluorophosphonate) (ALDRIDGE, 1953 a; MOUNTER and WHITTAKER, 1953).

That a fatty acid ester may be hydrolysed by several esterases, and that generally a complex mixture of esterases is present in animal tissues, has been shown at numerous occasions. It was also found a.o., that two fractions obtained from a culture of *Aspergillus oryzae*, active on the same substrate (p-nitrophenyl acetate) can be separated from each other by paper electrophoresis (WOODS and GILLESPIE, 1953 a) and by fractionation (WOODS and GILLESPIE, 1953 b). DELCOURT and DELCOURT (1953) separated two fractions active on naphthyl laurate from extracts of dog pancreas. Also seed lipase preparations from *Nigella sativa* were separated into two protein fractions by BAMANN and TIETZ (1953). It has been difficult to determine the range of specificity of the various esterases (MYERS and MENDEL, 1953). Moreover, the difference between ali-esterase and lipase has not been clearly defined. Frequently it is assumed that a high ali-esterase- and a low lipase activity of animal tissues (MYERS and MENDEL,1953; NACHLAS and SELIGMAN, 1949 and the reviews by AMMON and JAARMA, 1950; ANDERSON, 1948; LONGENECKER, 1946 and BLOOR, 1943) and of micro-organisms (COHEN *et al.*, 1953; CUTCHINS *et al.*, 1952; LUBERT *et al.*, 1949 and COLLINS and HAMMER, 1934) are due to one and the same enzyme. For several years bacteriologists have used esterolytic activity as a characteristic in the classification of species. A number of studies has been published showing that certain micro-organisms are capable to hydrolyse fats. Esterase activity of bacteria has been chiefly tested with natural substrates as butterfat, beef tallow or vegetable oils, however, more from the viewpoint of the bacteriologist than from that of the biochemist.

Until recently the function of the bacterial esterases in metabolic processes has got little attention. MENDEL and co-workers (1953) and RUYS (1954) were the first to show *in vitro* the inhibiting action of minute amounts of specific ali-esterase inhibitors on the growth of *Mycobacterium tuberculosis* in Dubos medium. It has also been published that a certain concentration of a substance that inhibits growth also inhibits bacterial esterase. Thus, according to SMITH and co-workers (1949), chloramphenicol inhibits the esterase activity of *Escherichia coli*. As further informations are not available it is not known whether these findings might be extended to other bacteria. Neither is it known why the esterases should be important for the growth of bacteria.

*Pseudomonas aeruginosa* has long been recognized as a fat-splitter ((VAN SOMMARUGA, 1894). The lipase from *Ps. aeruginosa* resists heating at 100° C. for five minutes (SÖHNGEN, 1911). Olive oil, triacetin and ethyl butyrate were hydrolysed by this bacterium (WALLS and CORPER, 1912). WALLS and CORPER believed that the same enzyme attacks all three esters. Tributyrin was also hydrolysed (MICHAELIS and NAKAHARA, 1923) and Tweens were hydrolysed by growing cultures of *Ps. aeruginosa* (SIERRA, 1957). It was noticed that a lipase preparation from *Ps. aeruginosa* showed a synthetic action when oleic acid and certain alcohols were present (VAN DER WALLE, 1927).

COLLINS and HAMMER (1934), LUBERT *et al.* (1949) and CUTCHINS *et al.* (1952) are of the opinion, that bacterial esterases are not specific. Notwithstanding the fact that bacteria attack a wide variety of esters, the possibility that the observed activity is due to several very similar, but more specific enzymes, has not been ruled out. Our experiences showed that the lipase and ali-esterase activities of *Ps. aeruginosa* were due to different enzymes.

It will therefore be expedient to consider certain difficulties in the work with esterases. Although the factors hydrolysing esters are fairly well known, their quantitative evaluation is still difficult, mainly due to technical (methodical) difficulties encountered in estimating lipase. One of the difficulties arises from the insolubility of most fatty substrates as well as from the typical retaining properties of the in water insoluble fatty acids that become free by the action of lipase.

As the fats and oils are insoluble in water, the rapidity of the hydrolysis will be dependent on the size of the fat or oil globules. In a very fine emulsion the rate of hydrolysis approximates that of the substrate in true solution (FRAZER and WALSH, 1933). GOLDMAN and RAYMAN (1952) showed that the rate of hydrolysis by bacteria is more dependent on the particle size of the emulsified fat than of the molecular composition of the triglyceridic component.

It is well-known that the titrimetric methods commonly used for the determination of lipolysis by bacteria do not give reproducible quantitative data. In many cases the variability of the size of the particles of the emulsified fat may play an important part. Moreover other causes may be conceived. The majority of the studies on esterases of micro-organisms were performed by adding an emulsified fat to a complex culture medium. However, this method is not suitable, because the results obtained can be influenced by other enzymatic activities. Many bacteria, because of their strong proteolytic nature, will not form enough acid from an ester to overcome the alkalinity produced from organic nitrogen (proteins, amino acids). Therefore, in this case it would be very difficult to detect lipase activity and in any case it would be impossible to estimate it quantitatively.

Manometric techniques valuable in estimating ali-esterase activity are not of much use in measuring lipase activity, because the insoluble free fatty acids do not readily liberate $CO_2$ from the bicarbonate buffer.

To arrive, in spite of these difficulties, at reliable information about the specificity and nature of the esterases of *Ps. aeruginosa*, two precautions were taken: a) an as large as possible surface of contact between enzyme and substrate was provided, b) the presence of any substance which could make the reaction mixture alkaline was avoided. The former requirement was met with by using water-soluble esters (Tweens) as substrate for lipase, and the latter by using resting cells.

## 2. CULTURE AND METHODS.

### a. **Culture.**

For these experiments was used a strain of *Ps. aeruginosa* isolated by Mr. ZAGT from a sample of water from the canal of the Mauritskade at Amsterdam. The behaviour of the micro- organism in routine tests will be reported elsewhere (SIERRA and ZAGT, 1958). The cultures were propagated by frequent transfers on peptone-agar slants; a fresh slant culture being prepared 18 to 20 hours before the inoculation of the liquid cultures.

### b. **Media.**

The media used in this work are common in most laboratories. For the sake of completeness they are listed below. They were sterilized by autoclaving for 20 minutes at 120° C.

1. Peptone-agar: Difco Bacto-peptone, 10 g; NaCl, 5 g; agar, 18 g; distilled water, 1000 ml. The pH was adjusted to 7.0–7.5.

2. Nutrient broth: Difco Bacto-peptone, 5 g; Liebig beef extract, 3 g; distilled water, 1000 ml. The pH was adjusted to 7.5.

### c. **Inoculation.**

As a rule the inoculation was carried out with one drop of a suspension of known density, made from a 20–24 hours old peptone-agar slant culture.

### d. **Growth conditions.**

The micro-organism was grown in submerged cultures at 37–38° C. by placing the culture flasks in a shaking machine which operates with 65 strokes of 20 cm per minute. Under these conditions the incubation was prolonged until harvesting.

If enzymatic activity had to be estimated, the cultures were grown in 200 ml of medium in 500 ml Erlenmeyer flasks. Growth of the bacteria was followed in 16.0 × 1.4 cm tubes with 5 ml of medium, by measuring the turbidity at intervals.

### e. **Estimation of growth.**

The turbidity of the cultures was periodically measured in duplicate, and expressed in extinction coeff. × 1000 ($E \times 10^3$). All photometric measurements were carried out with a red filter.

### f. **The source of enzyme.**

To measure esterase activity, washed-cell suspensions (with a known amount of bacteria) or cell-free enzyme preparations were used.

#### 1. Washed-cell suspensions.

At the end of the incubation period, cells were collected from the nutrient medium by centrifuging (5000 r.p.m. during 40 minutes), washed twice and then a thick suspension was made in water. These suspensions were adjusted by dilution to a suitable turbidity. Unless noted otherwise, the bacterial dry weight (D.W.) of these suspensions was 1 mg per ml. Washed-cell suspensions were prepared freshly every day, except for certain experiments that were stored in the refrigerator at 2–5° C. The D.W. of the suspensions was calculated from the turbidity by comparing this with the turbidity of suspensions of known D.W.

### 2. Cell-free preparations.

Cells were grown on nutrient broth in the usual way, harvested after five days by centrifuging at 5000 r.p.m. and then washed to remove the excess of nutrients. Active solutions of the enzyme were prepared by mixing 1 g of bacterial paste with 10 g of powdered glass, and 2.5 ml of saline solution and then cooled in ice water. About 5 g portions of this mixture were transferred to a chilled mortar and ground vigorously for five minutes to effect disruption. After centrifuging, 25 minutes at 15000–20000 r.p.m., 1 ml of the clear supernatant liquid (pale yellow) was used for esterase estimation by means of the Warburg apparatus, or titrimetrically. 1 ml of the active preparation is estimated to correspond with about 0.06 g of fresh wet cells.

Other techniques were tried and also cells were harvested after different incubation periods, but the most active preparations were obtained under the conditions mentioned above.

To measure the esterase activity present in the culture medium (excreted during the growth of the bacteria) the whole culture was neutralized after removing the cells by centrifuging for 30 minutes at 20000 r.p.m. 1 ml of the supernatant was used for each determination

## g. **Measurement of esterase activity.**

All determinations were made with a Warburg apparatus at 37.5 ± 0.05° C., or, when a titrimetric method was used, in a shaking machine at 37–38° C. Details of each method are outlined below.

### 1. Manometric determination.

When low fatty acid esters, for instance tributyrin, triacetin, or ethyl acetate were used as substrate, esterase activity was measured manometrically with a Warburg apparatus. As this technique is well known (UMBREIT, BURRIS and STAUFFER, 1951) it will not be described in detail here. It was first applied to esterase determinations by RONA and LASNITZKI (1924), and modified by AMMON (1933). AMMON's modification was used together with the controls and precautions described by STURGE and WHITTAKER (1950), and WHITTAKER (1951).

Anaerobic conditions were obtained by using an aqueous medium containing 0.025 M sodium bicarbonate, saturated by a gas stream of nitrogen with 5% $CO_2$ to obtain a pH 7.4. The total volume of the mixture was 5 ml, 0.5 ml of substrate in the sidearm in such concentration to give the desired final concentration, and 1 ml of enzyme source. After the cell suspension or cell-free preparation, the buffer and the substrate had been equilibrated at 37.5 ± 0.05° C. for 30 minutes, the ester solution was added. Readings were made at intervals of 30–35 minutes.

Water soluble esters, e.g. ethyl acetate, triacetin or methyl butyrate were dissolved directly in the bicarbonate buffer. The poorly soluble ester tributyrin was prepared as a 2.0% bicarbonate buffer emulsion with 1.0% gum acacia (arabic).

The enzyme activity was calculated from the initial rate of ester hydrolysis, which was constant during the period of measurement – provided that a diluted concentration of enzyme was used and the concentration of the substrate was large enough to maintain the enzyme saturated –. A final concentration of substrate of 2.0% was suitable for more active samples.

When inhibition experiments were carried out, the esterase inhibitors were incubated together with the enzyme in the bicarbonate buffer for 60 minutes at 37.5 ± 0.05° C. before the substrate was added. The percentage of inhibition was calculated after 30 minutes by comparison with the esterase activity of a control without inhibitor.

Differences of less than 5% were interpreted as being within the experimental errors.

### 2. Titrimetric determination.

When high fatty acid esters were compared with low fatty acid esters as substrates, both esterase activities were measured titrimetrically.

In the method to be described sterile 40 ml aluminium screw-capped bottles were used as reaction flasks. The total volume of the reaction mixture was 3 ml; 1 ml of enzyme preparation, and 2 ml of substrate. The mixture was adjusted to pH 7.8–8.0.

When esters of the high fatty acids – Tweens – were used, the mixture was continually shaken and was kept at 37–38° C. during a certain incubating period – generally 4 and 24 hours –. At the end of each incubation period the flasks were heated in a 70° C. bath after adding 10 ml of neutralized isopropanol and two drops of a 1% alcoholic solution of phenolphthalein. When esters of water soluble fatty acids -- tributyrin, triacetin and ethyl acetate – were used, the method was the same as above, except that 10 ml of water were used instead of isopropanol. This treatment proved to be the most efficient. The quantity of fatty acid formed was determined by titration with 0.1 N aqueous NaOH solution – carbonate free –, which was added with a micro-buret graduated to deliver volumes as small as 0.01 ml.

In the control experiments the enzyme preparation was heated at 100° C., until the esterase activity was completely destroyed. After heating the preparation was cooled and restored to the original volume.

Titrations were made in duplicate, giving identical values in most cases. If this was not the case we took the average value of the duplicates. The difference in titration values between the active mixture and the control was regarded as due to esterase. The results in $\mu$l NaOH 0.1 N per mg D. W. are plotted against time.

When inhibition experiments were carried out, esterase inhibitors were incubated together with the active sample in the reaction flask for 60 minutes at 37–38° C. before the substrate was added. The percentage of inhibition was calculated in the same way as in the manometric method.

The substrates were eight pure esters of fatty acids of different carbon-chain lengths ($C_2$, $C_4$, $C_{12}$, $C_{16}$, $C_{18}$). Water soluble esters: ethyl acetate, methyl butyrate, triacetin, Tween 20, Tween 60 and Tween 80. They were dissolved directly in sterile water. Tributyrin was prepared as an aqueous emulsion with gum acacia. All esters were sterilized at 110° C. for 20 minutes before they were dissolved.

All Tweens are esters of a polyoxyalkylene derivative of sorbitan (Atlas Surface Active Agent, 1948). They are lauric acid ($C_{12}$) in Tween 20, palmitic acid ($C_{16}$) in Tween 40, stearic acid ($C_{18}$) in Tween 60, and oleic acid ($C_{18}$) in Tween 80.

Each substrate was used at a concentration above that required to produce maximum titrable acidity with a given suspension of cells or an enzyme preparation.

Tweens and simple esters were used without being subjected to any further purification ¹).

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¹) Tweens were obtained from the Atlas Powder Co., Wilmington, Delawere. Triglycerides and simple esters from the British Drug Houses Ltd., Poole, England.

### h. **Inhibitors.**

The esterase inhibitors used were: diethyl p-nitrophenyl phosphate (E 600, paraoxon), sodium fluoride, sodium arsenilate (atoxyl), sodium taurocholate, sodium oleate, and physostigmine (eserine). They were dissolved or suspended in distilled water and neutralized to pH 7.2–7.4 when necessary.

When E 600 was added to the nutrient medium, the solution of this inhibitor was sterilized by filtration.

The solution of the inhibitors was kept in the refrigerator at 2–3°C.

## 3. RESULTS.

In preliminary experiments the presence of esterase activity in *Ps. aeruginosa* was detected by means of plate methods. The test of the hydrolysis of beef tallow was done according to the original method of EIJKMAN (1901). Olive oil, corn oil and butterfat were tested with the method of KNAYSI (1941). Tributyrin was tested according to the ANDERSON test (1934). The bacteria exhibited a strong esterolytic activity on all fatty substrates tested.

Besides on the substrates mentioned, the bacterium was also tested on triacetin and ethyl acetate. This was done by the potentiometric technique with the glass electrode. It is sufficient to note that esterase activity was detected by changes in pH of the reaction mixture starting at pH 8.0. As source of enzyme the flask contained 1 ml of a cell-suspension (1 mg D.W. per ml) collected from a two days old nutrient broth culture. The experiments showed a very low activity of the bacteria towards these substrates. This confirms findings in earlier work.

By examining – titrimetrically – washed cells harvested from nutrient broth, it was found that the Tweens were all attacked at the same rate except Tween 20, which was hydrolysed more rapidly. These results are reproduced graphically in Fig. 1.

### a. **Selective inactivation of the esterase activities by heat.**

When we used the titrimetric method to measure the inactivation by heat, it was found that a control containing heated cells (15 min. at 100° C.) had still a residual lipase activity when measured on Tween 80, while the activity on tributyrin was completely destroyed by this treatment (see Table 1).

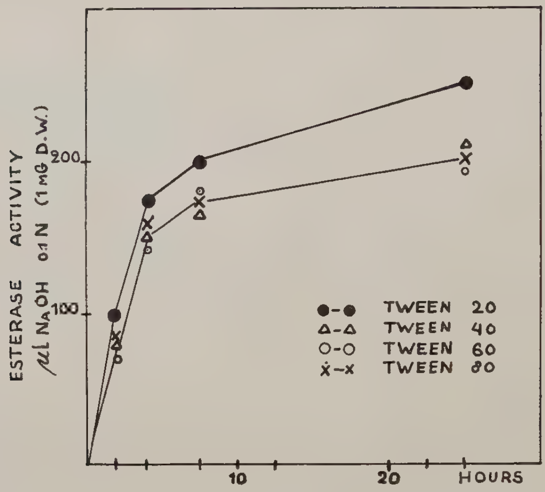

Fig. 1. Hydrolysis of Tweens by washed-cells. Age of the cells: 5 days.

That the residual lipase activity on Tween 80 shown by heated cells means a true activity, is indicated by the controls (Exp. 1 and 2).

These show that a possible spontaneous hydrolysis of the Tween 80 as well as any change in the acidity of the bacterial suspension were negligible during the period of measuring. In view of these facts a selective inactivation of the enzymes was attempted.

The thermal stability of the enzymes responsible for these activities was measured by heating aliquots of known unbuffered aqueous washed-cells suspensions – or enzyme solutions – at pH 7.0 to various temperatures between 50 and 100° C. during different periods. After heating the samples were cooled and restored to the original volume if necessary. The degree of esterase inactivation in relation to the heating-time was followed on Tween 80, tributyrin, triacetin and ethyl acetate.

It was found that the activities on ethyl acetate and triacetin were both completely stopped by exposure for 15 min. to 70° C., while the lipase (on Tween 80) and tributyrinase remained active. On the other hand, tributyrinase was completely inactivated when submitted to 100° C. for 15 min., whereas lipase remained active. A heating-time of 60 min. (at 100° C. )was necessary for complete inactivation of the lipase. The difference in thermostability shows that there are at least three activities responsible for the hydrolysis

TABLE I.

Esterase activity[1]) ($\mu$l NaOH/mg D.W.) of living and heated bacteria.

Cells were harvested from nutrient medium after five days of cultivation. Controls contained cells heated for 15 min. to 100° C.

| Incubation time (hrs) | Exp. 1<br>no bacteria<br>Tween 80 | Exp. 2<br>living bacteria<br>no substrate | Exp. 3<br>living bacteria<br>Tween 80 | Exp. 4<br>heated bacteria<br>Tween 80 | Exp. 5<br>no bacteria<br>tributyrin | Exp. 6<br>living bacteria<br>tributyrin | Exp. 7<br>heated bacteria<br>tributyrin |
|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 100 | 50 | 0 | – | 0 |
| 4 | 0 | 0 | 150 | 60 | 0 | 200 | 0 |
| 6 | 0 | 0 | 150 | 100 | – | – | – |
| 24 | 0 | 0 | 200 | 100 | 60 | 510 | 60 |

[1]) The esterase activity on tributyrin is not corrected for the spontaneous hydrolysis of the substrate.

of Tween 80, tributyrin, triacetin and ethyl acetate *viz.*, lipase, tributyrinase and still one or two activities.

In estimating the activity of the lipases hydrolysing Tween 20, 40 and 60 ($C_{12}$, $C_{16}$, $C_{18}$) it was found that all had the same thermostability (see Table 2).

TABLE 2.

Inactivation of the lipase of *Ps. aeruginosa* by heat.

Lipase activity (μl NaOH 0.1 N/mg D.W./24 hrs) on Tween was measured and the percentage of inactivation was calculated by comparison with the activity of intact cells.

| Substrate | Amount of lipase activity remaining after exposure of the cells for 15 min. to 100°C. (%) |
|---|---|
| Tween 80 | 75 |
| Tween 60 | 75 |
| Tween 40 | 75 |
| Tween 20 | 70 |

As table 2 shows that the thermostability of the lipases hydrolysing Tween 20, 40 and 60 is about the same as that of the lipase hydrolysing Tween 80, it seems likely that there is only one enzyme that hydrolyses the Tweens. For the same reason there seems to be only one enzyme that hydrolyses triacetin and ethyl acetate.

As the lipase is very thermostable and the action on tributyrin, triacetin and ethyl acetate is easily destroyed, it is clear that lipase does not hydrolyse these substrates. It was due to these findings that we could study both activities separately. Thus, in future experiments with lipase the source of enzyme will consist of bacteria – or cell-free preparations – heated for 15 min. at 100° C.

Ali-esterase will be measured with fresh cells – or cell-free preparations –. It should be mentioned that washed cells collected after 5 days of incubation did not show any activity on triacetin and ethyl acetate (Figs 5 and 6).

### b. Growth and enzyme production.

The activities of individual esterases, as shown by washed cells, were compared with the growth.

Flask-cultures were removed from the shaker at intervals and harvested. The cell suspension was standardized and examined for esterase activity. Variations of the esterase activity with the "age" of the culture of a standard mass of cells are given in Figs 2, 3, 4 and 6.

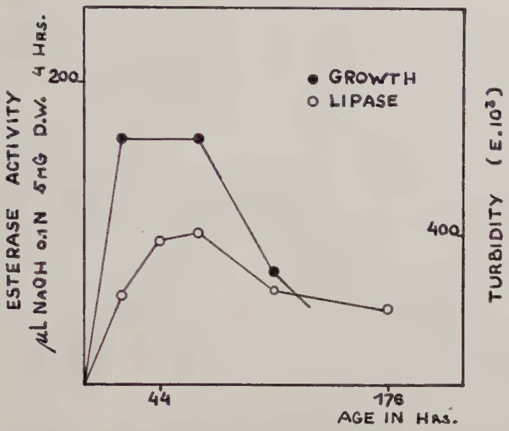

Fig. 2. Variation of lipase activity of washed-cells of *Ps. aeruginosa* with age of the culture. Substrate: Tween 80. Washed-cells were exposed for 15 min. at 100° C.

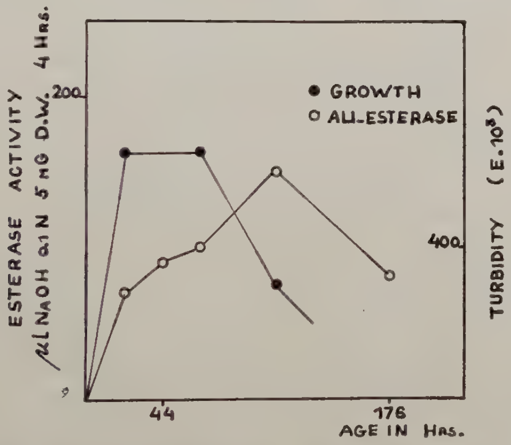

Fig. 3. Variation of ali-esterase activity of washed-cells of *Ps. aeruginosa* with age of the culture. Substrate: tributyrin.

Lipase (Fig. 2) started low, passed through a maximum during the stationary phase, and then declined slowly.

The activities on ethyl acetate and triacetin show a parallel behaviour which indicates that triacetinase is responsible for the hydrolysis of these two esters (Figs 5 and 6). However, this activity is different from the lipase and tributyrinase because triacetinase could only be demonstrated in the first stage of the growth.

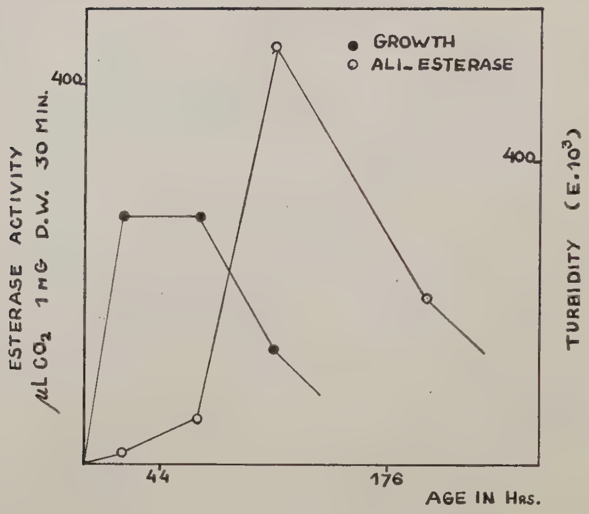

Fig. 4. Variation of ali-esterase activity of washed-cells of *Ps. aeruginosa* with the age of the culture. Substrate: tributyrin (analogous variation as that in Fig. 3, but here ali-esterase was estimated manometrically).

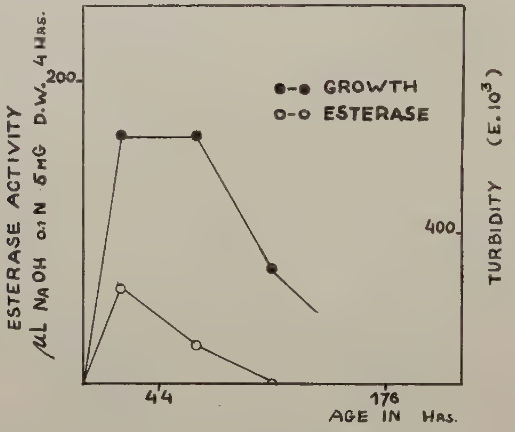

Fig. 5. Variation of ali-esterase activity of washed-cells of *Ps. aeruginosa* with the age of the culture. Substrate: ethyl acetate.

A peculiar variation shown by tributyrinase is represented in Figs 3 and 4. These figures show that tributyrinase starts and increases slowly until the stationary phase. Notwithstanding also a decay of the enzyme is to be expected, the highest activity is not found before the cultures are 4–5 days old. In the enzyme Type II variation (GALE, 1952) cells taken early in the growth period have little or no activity. The enzyme is formed during the growth, reaching a maximum activity at about the time of cessation of cell division. After the end of the growth the activity may fall, due to death of the cells, oxidation or digestion of the enzyme protein, etc.

This type of variation we have found with ali-esterase of *Mycobacterium phlei*, *Bacillus licheniformis*, and *Micrococcus pyogenes* var. *aureus* (SIERRA, 1955).

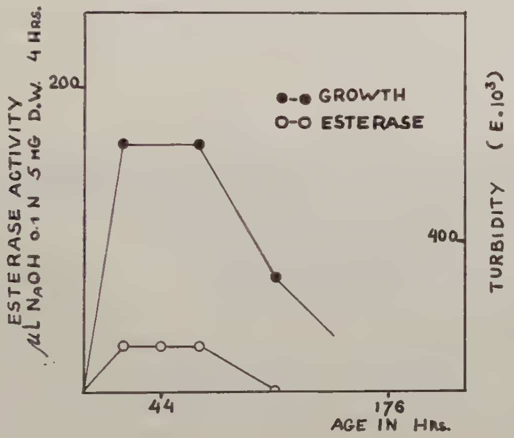

Fig. 6. Variation of ali-esterase activity of washed-cells of *Ps. aeruginosa* with the age of the culture. Substrate: triacetin.

It was observed that tributyrinase activity of *Ps. aeruginosa* rises with the clearing of the nutrient broth culture (between 4–5 days). That this decrease in turbidity (shown in growth curves in the Figs 2, 3, 4, 5 and 6) represents a lytic phenomenon in which the cells are destroyed will be shown elsewhere (SIERRA and ZAGT, 1958).

These observations suggest that the tributyrinase as an endocellular enzyme is released by lysis of the cells.

By fractioned centrifugation of a partially lysed culture it was possible to show that the whole tributyrinase activity is associated with those cells which are more or less affected by lysis, but not with the cell debris. Washed-cells collected by centrifuging at 5000 r.p.m. showed a high esterase activity, while the cell debris gained from the still turbid supernatant by centrifuging at 20000 r.p.m. had very little or no esterase activity.

### c. **Liberation of tributyrinase from the cell.**

As previously noted, a high tributyrinase activity was found after 5 days of incubation, when lysis of a high percentage of the population was shown by the clearing of the cultures. Nevertheless, it is not possible to draw a definite conclusion about the absolute endocellular nature of the enzyme, because tributyrinase as well as lipase were present too in a cell-free supernatant of very young broth cultures (Table 3).

However, also in very young cultures lysis is not excluded, because even there we observed quite a number of lysed cells (Fig. 7).

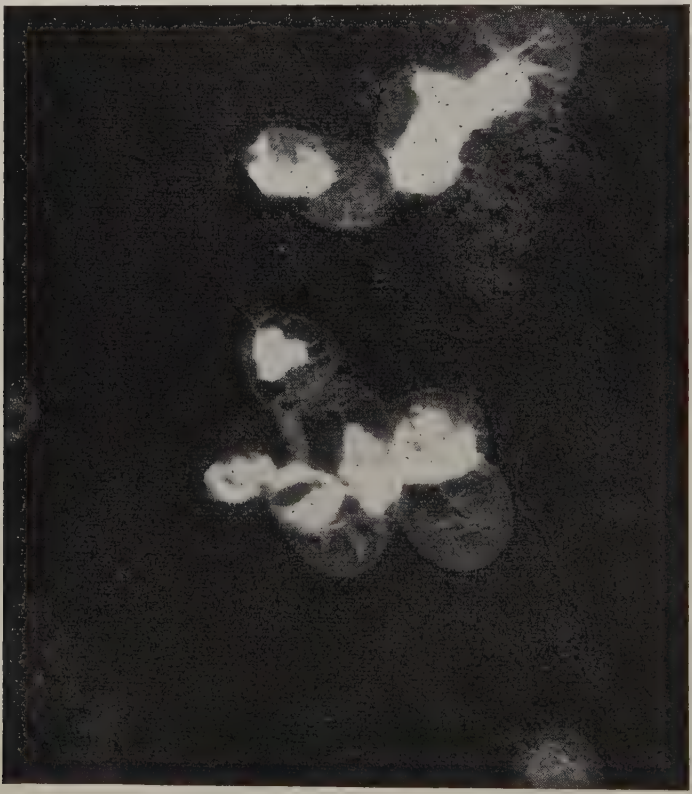

Fig. 7. Electron micrograph showing lytic zones in bacteria from one day old nutrient broth culture of *Ps. aeruginosa* (25000 ×). The culture was centrifuged at 20000 r.p.m. during 20 min., washed twice and resuspended in water.

TABLE 3.

Lipase and ali-esterase activities in cell-free cultures.
Culture medium: nutrient broth.

| Age of the culture (days) | Lipase activity ($\mu$l NaOH 0.1 N/ml/24 hrs) Substrate: Tween 80 | Ali-esterase activity [1] ($\mu$l NaOH 0.1 N/ml/24 hrs) Substrate: tributyrin |
|---|---|---|
| 0 | 0 | 0 |
| 1 | 120 | 170 |
| 5 | 140 | 200 |

[1] Corrected for spontaneous hydrolysis of the substrate.

TABLE 4.

Comparison of the results achieved with two techniques of preparation of cell-free esterases.

1 ml of enzyme solution corresponds with 0.06 g of wet cells. Lipase activity was measured with the enzyme solution after exposure for 15 min. to 100° C.

| Technique | Shaking time (hrs) | Ageing time (days) | Lipase activity ($\mu$l NaOH 0.1N/ml/24 hrs) Substrate: Tween 80 | Ali-esterase activity [1] ($\mu$l $CO_2$/ml/30 min.) Substrate: tributyrin | triacetin |
|---|---|---|---|---|---|
| Shaking with small glass beads in the Microid Flask Shaker | 3 | 3 | 170 | 90 | not split |
| | 5 | 3 | 150 | 85 | ,, ,, |
| Hand grinding (5 min.) | – | 5 | – | 243 | ,, ,, |

[1] Corrected rate.

In order to investigate this further it seemed indicated to liberate the enzymes from the cell by stimulating lysis. However all attempts to stimulate autolysis of young cells failed (SIERRA and ZAGT, 1958).

An active cell-free enzyme solution was prepared by shaking a washed bacterial paste with glass beads at 500 oscillations per minute for three hours at room temperature. A heavy suspension of the bacteria in saline containing 2 g of wet weight was mixed with 10 g of glass beads and shaken in capped aluminium flasks in a Microid Flask Shaker [1].

After 3 hrs the cell debris were extracted with a saline solution. By centrifuging at 20000 r.p.m. one gets a clear, pale yellow supernatant.

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[1] Griffin & Tatlock Ltd., London.

TABLE 5.

Survey of the relative inhibiting power of inhibitors of pancreas and liver esterase.

| Inhibitor | Relative potency of inhibition | | References |
|---|---|---|---|
| | Lipase (pancreas) | Ali-esterase (liver) | |
| Na-fluoride | + | ++++ | Loevenhart & Pierce (1906), Rona & Haas (1923), Nachlas & Seligman (1949). |
| Atoxyl | + | +++ | Rona & Pavlovic (1923), Rona & Bach (1920), Nachlas & Seligman (1949), Myers & Mendel (1953). |
| Na-taurocholate | activation | ++++ | Willstätter & Memmen (1924), Gomori (1945), Nachlas & Seligman (1949). |
| Na-oleate | activation | ++++ | Bamann & Leaverenz (1934). |
| Eserine | + | + | Nachlas & Seligman (1949). |
| Paraoxon | +++ | +++++ | Myers (1955) personal communication, Myers & Mendel (1953). |

Both lipase and ali-esterase were present in this solution as is shown in table 4, which also shows that shaking during longer periods did not yield more active solutions.

Attempts to find a way to get more active enzyme preparations were carried out. The hand grinding technique as described earlier was the most efficient way. Cell-free lipase and ali-esterase solutions were prepared and it was found that the tributyrinase activity was nearly half that of intact cells (see table 4). Cell-free extracts remained active for at least one year when kept at –25° C. or as lyophilized material.

It should be noted that in these solutions the same thermostability for lipase and ali-esterase was found as earlier in washed-cells suspensions.

### d. **Chemical inhibition of esterases.**

The study of the esterases from animal tissues has been mainly done by following the efficiency with which various substrates could be hydrolysed and the effect of specific inhibitors on their activity.

In some cases advantage may be taken of the fact that an inhibitor – or activator – acts on the activity of one enzyme but not on that of another, thus differentiating them. Sodium fluoride, sodium arsenilate, sodium taurocholate, sodium oleate, physostigmine and diethyl p-nitrophenyl phosphate are well-known esterase inhibitors. The relative inhibiting action of these compounds on a given type of esterase as well as their literature references are given in table 5.

Table 6 presents the effect of these inhibitors on the esterase activities of *Ps. aeruginosa.*

TABLE 6.

Effect of specific esterase inhibitors on the lipase and ali-esterase activity of *Ps. aeruginosa.*

1 ml of washed cells containing 1 mg D.W. was incubated at 37—38°C. for 60 minutes (prior to the addition of substrate) in presence and absence of esterase inhibitors. After the substrate was added the mixture was again incubated for 3 hrs and then the esterase activity was estimated by titration. The washed-cells used for lipase estimation were exposed for 15 min. to 100°C.

| Inhibitor | Concentration 1) (M) | Lipase activity | | | | ali-esterase activity | | |
|---|---|---|---|---|---|---|---|---|
| | | Inhibition on the substrate: (%) | | | | | | |
| | | T. 80 ($C_{18}$) | T. 60 ($C_{18}$) | T. 40 ($C_{16}$) | T. 20 ($C_{12}$) | tributyrin | triacetin | ethyl acetate |
| Na-fluoride | $5.0 \times 10^{-2}$ | 0 | 0 | 0 | 0 | 0 | 89 | 98 |
| Atoxyl | $2.0 \times 10^{-2}$ | 0 | 0 | 0 | 0 | 0 | 56 | 70 |
| Eserine | $5.0 \times 10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Na-taurocholate | $1.0 \times 10^{-2}$ | 20 | 20 | 20 | 18 | 65 | 100 | 100 |
| Paraoxon | $1.8 \times 10^{-4}$ | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Na-oleate | $4.1 \times 10^{-3}$ | 0 | 0 | 0 | 0 | 100 | — | — |
| Tween 80 | 2) | | | | | 80 | | |

1) The concentration of inhibitor in the mixture during the pre-incubation period (before the substrate was added) was twice these values.

2) Tween 80 compound of unknown molecular weight was used in a concentration of 0.6% and twice in the pre-incubation period.

Lipase as well as the two ali-esterases are insensitive to eserine at the concentration tested, but all are sensitive to a low concentration of paraoxon. Paraoxon in a concentration of $1.8 \times 10^{-4}$ M completely inhibits the activity of the three esterases. Lipase and tributyrinase are insensitive to fluoride, while triacetinase is sensitive. Lipase is atoxyl resistant, whereas triacetinase and tributyrinase – the latter in a smaller degree – are sensitive to this inhibitor. Taurocholate inhibits both lipase and tributyrinase, but in a different degree.

In table 7 data of inhibition of tributyrinase of washed-cells and cell-free enzyme solutions are compared.

TABLE 7.

Comparison of the effect of ali-esterase inhibitors estimated with washed-cells and cell-free enzyme solutions.

Washed-cells suspensions (1 mg D.W.) or 1 ml of cell-free enzyme solutions — gained by the hand grinding technique — were incubated for 60 min. to 37.5 ± 0.05° C. in presence and absence of ali-esterase inhibitors in the bicarbonate buffer. After adding the substrate, ali-esterase activity was measured at intervals of 30 min. and the percentage of inhibition was calculated by comparison with ali-esterase activity of the blancos without inhibitor.

| Inhibitor | Concentration (M) | Ali-esterase inhibition (%) | |
|---|---|---|---|
| | | substrate: tributyrin | |
| | | washed-cells | cell-free enzyme solution |
| Na-fluoride | $5.0 \times 10^{-2}$ | 0 | 0 |
| Eserine | $5.0 \times 10^{-5}$ | 0 | 0 |
| Atoxyl | $2.0 \times 10^{-2}$ | 40 | 32 |
| Na-taurocholate | $1.0 \times 10^{-2}$ | 73 | 100 |
| Paraoxon | $1.8 \times 10^{-4}$ | 100 | 100 |
| | $1.8 \times 10^{-5}$ | 85 | |
| | $1.8 \times 10^{-6}$ | 50 | |
| Na-oleate | $2.3 \times 10^{-2}$ | 100 | 100 |
| Tween 80 | ¹) | 100 | 81 |

¹) Tween 80 was used in a concentration of 0.6%.

From table 7, it can be seen that the results of the experiments with both sources of enzyme are in good accordance.

Finally a summary of the inhibition experiments is presented in table 8.

### e. Action of paraoxon upon growth.

These experiments were carried out with paraoxon which appeared to be the most potent inhibitor of the esterases of *Ps. aeruginosa* (see table 6).

Table 9 shows that concentrations of paraoxon amply sufficient to suppress all esterase activity ¹), added to the nutrient medium

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¹) In washed-cell suspensions harvested from an one day old nutrient broth paraoxon culture not any esterase activity on tributyrin could manometrically be detected.

TABLE 8.

Summary of the relative inhibiting power of specific inhibitors of esterases of *Ps. aeruginosa.*

| Inhibitor | Lipase | Tributyrinase | Triacetinase |
|---|---|---|---|
| Eserine | — | — | — |
| Atoxyl | — | — | +++ |
| Na-fluoride | — | — | ++++ |
| Na-taurocholate | + | +++ | ++++ |
| Paraoxon | ++++++ | ++++++ | ++++++ |
| Tween 80 | | ++++ | |
| Na-oleate | — | +++ | |

Designation of the symbols: — no effect; +, ++, +++, etc. different degrees of inhibition.

TABLE 9.

Effect of paraoxon upon the growth of *Ps. aeruginosa* in nutrient broth. Temperature of incubation: 37—38° C. Concentration of paraoxon in culture medium: 80 mg per 100 ml.

| Incubation time (days) | Growth ($E \times 10^3$) | |
|---|---|---|
| | nutrient broth | nutrient broth + paraoxon |
| 1 | 140 | 112 |

proved to be unable to arrest the growth of the bacteria at 37° C.

Because of the endocellular nature of the esterases, experiments under other conditions were done *viz.*, addition of low concentrations of Tween 80 (Ruys, 1954) or growth at lower temperatures, but all attempts to suppress the growth of *Ps. aeruginosa* by inhibiting esterase activity failed.

## 4. Discussion.

Esterase activity seems to be the result of the liberation of materials from the cell by lysis, since certain amounts of esterases could be extracted from the cell and the activity of washed cells did increase during the phase of decreasing bacterial population.

The evidence for the presence of three esterases in *Ps. aeruginosa* seems convincing and is mainly based on the following observations:

(a) Differences in thermolability.
(b) Differences in substrate specificity.
(c) Differences in the relation between growth and activity.
(d) Differences in behaviour to inhibition.

Lipase and ali-esterase of *Ps. aeruginosa* show a higher specificity than was ascribed to bacterial esterases up till now. Lipase hydrolyses high fatty acid esters ($C_{12}$, $C_{16}$, $C_{18}$) but not tributyrin ($C_4$), triacetin and ethyl acetate ($C_2$). Tributyrinase hydrolyses tributyrin ($C_4$) but does not split triacetin and ethyl acetate ($C_2$). Triacetinase appears to be specific for a chain of the length of two carbons, since it splits only triacetin and ethyl acetate.

The observation that Tween 80 and sodium oleate act selectively as inhibitors of tributyrinase provides an important means of differentiation between ali-esterase and lipase. Natrium oleate has no action at all upon lipase, and Tween 80 itself is a substrate for lipase. Special attention is drawn to the specific ali-esterase inhibiting action of Tween 80, sodium taurocholate and sodium oleate, because of the surface active properties of these inhibitors and of the endo-cellular nature of the esterases. It might be conceived that this action could be due to changes in the cell wall permeability induced by these inhibitors but this can not be the cause since the inhibitory action on tributyrinase activity by these surface active agents was also found when a cell-free enzyme solution was used (table 7).

Later experiments indicate that the inhibitory action of Tween 80 towards the ali-esterase may be due to the inhibiting action of the oleic acid – or oleate – that becomes free by the action of the lipase.

This is substantiated by the fact, that Tween 80 does not show any action on the ali-esterases of other bacteria *viz.*, *Bacillus mycoides* and *Bacillus licheniformis* as these do not possess any lipase.

It is supposed that the hydrolysis of the four Tweens is due to the same enzyme, because in all four cases the same inactivation by heat is found. This assumption is considerably supported by the results shown in table 6. The same degree of inhibition was found for lipase on all Tweens with each of the inhibitors. Also it appeared to be possible that it is the same enzyme which hydrolyses triacetin and ethyl acetate, because we found the same inactivation by heat and the same relationship between growth and activity. However, virtually the same inhibition of ali-esterase was shown when it acted on triacetin or on ethyl acetate. Moreover the sensitivity of the triacetinase to fluoride and atoxyl, compared with the insensitivity of lipase and tributyrinase, shows once again that triacetinase is a separate enzyme.

Since one of the most characteristic ali-esterases is that of the liver, and the most typical lipase comes from the pancreas, it seemed interesting to compare their activities with bacterial ali-esterase and lipase. By comparing tables 5 and 8 it can be seen that they are not identical. Lipase of *Ps. aeruginosa* is inhibited by taurocholate and by very low concentrations of paraoxon and it is not significantly affected by oleate, and also has a narrower specificity pattern.

Bacterial tributyrinase, in contrast to the liver enzyme, is practically not affected by fluoride and atoxyl and has a very narrow specificity pattern.

The results collected in table 9 indicate that the growth of *Ps. aeruginosa* in nutrient broth could not be affected by inhibiting its esterases. We also tested the effect of very high concentrations of paraoxon (80 mg per 100 ml) on other bacteria e.g., *Bacillus mycoides*, *Bacillus licheniformis*, and *Staphylococcus aureus* Oxford which also contain ali-esterase (SIERRA, 1956); in no case did we observe any significant inhibition of growth in the nutrient broth. It is interesting to note that in Warburg experiments 5 mg per 100 ml of paraoxon was sufficient for a complete inhibition of the most active bacterial ali-esterases, notwithstanding the concentration of the cells in experiments with washed-cells suspensions was many times greater than that in growing cultures. As appears from the relationship between ali-esterase activity and age of the culture (Figs 2, 3, 4, 5 and 6) we may conclude that the ali-esterases are of no importance for the growth processes.

As *in vitro* ali-esterases of some bacteria can be inhibited completely without any observable morphological change as f.i. loss of motility, etc., and inhibition of ali-esterase does not or very slightly affect the growth of some bacteria, our finding fails to support MENDEL's suggestion (MENDEL *et al.*, 1954) that ali-esterases play an essential rôle in the metabolism of lower forms of life.

## Summary.

1. A method for the estimation of lipolytic activity of bacteria is described. It is based on the application of the water soluble Tweens as substrate for lipase.

2. The esterolytic power of a strain of *Ps. aeruginosa* was studied with five esters of different fatty acids with different length of chain. Three esterases were identified and estimated as separate

entities during the growth of the bacteria in nutrient broth. They showed their existence by their differences in sensitivity towards elevated temperature and towards specific inhibitors, showing a distinct specificity pattern.

The hydrolysis of long chain fatty acid esters ($C_{12}$, $C_{16}$, $C_{18}$) is due to an enzyme which is usually designated as lipase. The hydrolysis of tributyrin is entirely due to an ali-esterase. The hydrolysis of triacetin and ethyl acetate is due to an ali-esterase other than tributyrinase. Lipase and tributyrinase of *Ps. aeruginosa* are not identical with pancreas and liver esterases. The quantitative variation of these enzymes with the "age" of the culture was studied. It was found that in ageing suspensions the higher tributyrinase activity coincides with the lysis of the culture. This finding suggests the endo-cellular nature of this enzyme. Tributyrinase and lipase were also liberated from the cell by grinding with powdered glass.

3. Concentrations of diethyl p-nitrophenyl phosphate (E 600) which are amply sufficient to suppress all esterase activity proved to be unable to arrest the growth of the tested bacteria. It seems therefore unlikely that ali-esterases play an essential rôle in the metabolism of bacteria.

## Acknowledgment.

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# LA FILTRATION DU VIRUS POLIOMYELITIQUE SUR FILTRES SEITZ; PHENOMENES D'ABSORPTION ET D'ELUTION DU VIRUS

par

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(Reçu le 13 Mai 1957).

L'introduction d'une double filtration du virus poliomyélitique sur filtre Seitz $S_1$ lors de la fabrication du vaccin antipoliomyélitique a présenté un facteur décisif pour la garantie d'innocuité du vaccin. En effet, il a été démontré qu'au cours de l'inactivation par le formol, les débris protéiques du milieu étaient susceptibles de former des conglomérats au centre desquels un virus pouvait échapper à l'action du formol et se retrouver à l'état vivant dans le produit final. Cette possibilité, liée à l'insuffisance des contrôles quant au virus vivant, est à la base des accidents survenus en 1955 aux Etats-Unis.

Actuellement, le milieu de culture riche en virus est filtré avant adjonction de formol et une seconde filtration est effectuée au cours de l'inactivation. Depuis l'introduction de ces normes de sécurité et de mesures plus strictes de contrôle, plus aucun lot n'a été trouvé positif et plus aucun incident post-vaccinal, lié à l'injection de vaccin, n'a été signalé.

Cependant, la nécessité de ces filtrations répétées sur filtres Seitz $S_1$ ont soulevé un problème quant à l'efficacité du vaccin. En effet, au cours de la filtration une certaine quantité de virus est absorbée par le filtre et ces pertes de virus s'accompagnent d'une diminution du pouvoir antigénique.

Au cours d'expériences réalisées dans notre laboratoire nous avons étudié les conditions de filtration du virus poliomyélitique sur filtres Seitz. Nous présentons dans cet article les conclusions auxquelles nous sommes arrivés et les facteurs nécessaires à la récupération du virus lors de la filtration.

## 1. ABSORPTION DU VIRUS.

Le phénomène de l'absorption du virus poliomyélitique sur filtres Seitz $S_1$ et sur filtres Corning U.F. a été signalé par la plupart des auteurs.

SALK (1955) observe dans certains cas une diminution de titre de virus de l'ordre de 50% après filtration (6.7 à 6.3) Certains lots de vaccin montrent une antigénicité faible et ce phénomène est attribué aux pertes de virus au cours de la filtration (lot B 2,1955). Le problème de l'efficacité maximale du vaccin représente certainement le souci essentiel de la fabrication américaine actuelle.

Mean of infectious titers before and after filtration of tissue culture fluids containing each of the three types of poliomyelitis virus used in the preparation of vaccine for field tests in the summer of 1954.

| | Type 1<br>Mahoney | | Type 2<br>MEF-1 | | Type 3<br>Saukett | |
|---|---|---|---|---|---|---|
| Vaccine Lots | Pre-Filt. | Post-Filt. | Pre-Filt. | Post-Filt. | Pre-Filt. | Post-Filt. |
| A-1 – A-9 | 6.83 | 6.92 | 6.21 | 6.10 | 7.16 | 7.06 |
| B-1 – B-9 | 6.72 | 6.39 | 6.42 | 6.31 | 6.46 | 6.32 |
| Average | 6.77 | 6.71 | 6.32 | 6.21 | 6.81 | 6.69 |

Titers are expressed as the negative log of the 50 per cent infectious end point for tissue cultures using 0.5 ml inoculum in roller tubes prepared with trypsinized monkey kidney cell suspension.

Le Dr H. VON MAGNUS nous a communiqué (communication personnelle) ne pas avoir remarqué de pertes significatives lors de la filtration du virus vivant mais bien lors de la filtration du virus formolé. Ces pertes de virus se manifestent par une baisse du pouvoir antigénique.

Dans notre laboratoire, dès le début de nos travaux concernant le vaccin antipoliomyélitique, nous nous sommes heurtés au problème de l'absorption du virus. Lors de la filtration d'un lot de virus non formolé de type $MEF_1$, titrant $10^{6.5}$ IDTC 50/0,5 ml, nous avons constaté la disparition quasi totale du virus. Le titre après filtration était de $10^{3.65}$, soit une absorption de 99% du virus. La figure 1 montre clairement la différence d'absorption notée avec un autre lot de virus (1342) où les conditions de filtrations ont été différentes.

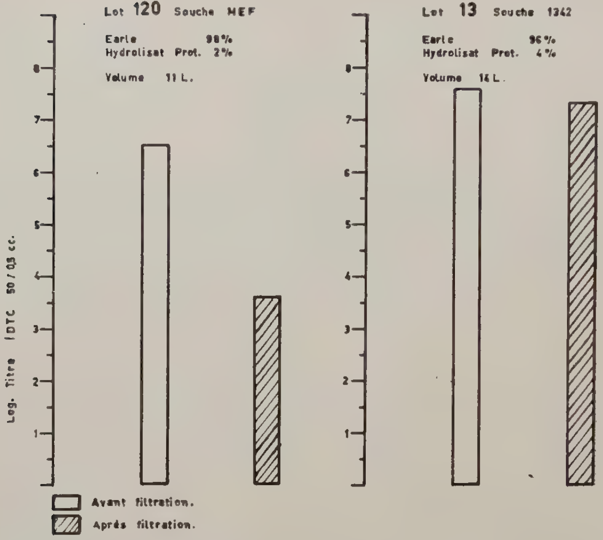

Fig. 1. Filtration sur Filtres Corning Fine, Ultra-Fine.

A la suite d'essais répétés de filtration nous avons acquis la conviction que deux facteurs pouvaient expliquer la différence des résultats: d'une part la teneur en acides aminés et d'autre part le volume filtré. En effet, dans le cas du virus 1342 la teneur en acides aminés est double de celle utilisée dans l'expérience avec le virus $MEF_1$, le volume filtré est de 14 L au lieu de 11 L. Dans une série d'essais nous avons étudié l'influence de ces deux facteurs sur la filtration du virus.

### 1. Volume.

L'importance de la quantité de liquide filtrée par unité de surface est certaine. Il apparaît qu'au cours de la filtration sur filtres Seitz les premières fractions montrent une absorption maximale. Ce phénomène est général pour tout filtre Seitz, et même pour les filtres clarifiants et non stérilisants de la série K. Après passage d'une certaine quantité de liquide, différente selon le type de filtre, le virus est récupéré quasi totalement dans le filtrat. Lorsque la filtration est poursuivie, le passage d'un volume excessif entraîne une obturation du filtre et une nouvelle rétention du virus. Comme exemple type de l'influence du volume filtré nous reprenons une expérience réalisée sur filtre Seitz $K_5$ et $EKS_1$ au moyen de virus Saukett:

Le titre de départ du virus est de $10^7$ IDTC 50/0,5 ml. Après passage de 1L sur filtre Seitz $K_5$ (13 cm Ø) le titre est de $10^{5.9}$, soit une absorption de plus de 90%. Les fractions ultérieures (2, 3, 4 et 5 L) ont un titre de $10^7$, le virus n'est donc plus absorbé. Lorsque ce même virus est filtré sur filtre $EKS_1$ de même diamètre, l'absorption du virus (de près de 99%) est manifeste jusqu'au 10ième Litre. Dans un échantillon du 15è L, le virus est récupéré quasi totalement. Au delà de 40 L la filtration se ralentit fortement et une nouvelle diminution du titre est observée.

Ce phénomène est illustré également dans l'expérience de la fig. 2. Après absorption dans la première fraction de 5 L de virus, la récupération est totale dans les deux premières fractions.

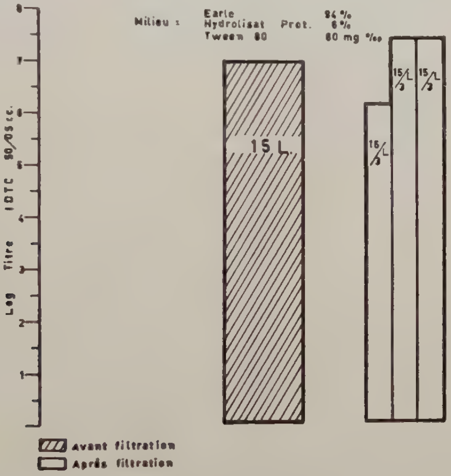

Fig. 2. Filtration de 3×5 Litres sur Filtre Seitz $EKS_1$ (Lot Saukett).

En conclusion de ces expériences, il est important de déterminer pour chacun des filtres Seitz, le volume adéquat nécessaire pour la filtration du virus poliomyélitique. Dans le cas du filtre $S_1$, ce volume critique est d'environ 250 cm³ par cm² de surface. Les auteurs américains recommandent également le passage d'un certain volume de liquide par unité de surface afin d'éviter les pertes de virus par absorption (SALK, 1957).

## 2. Acides aminés.

Le problème de l'absorption du virus poliomyélitique lors de la filtration s'est posé d'une façon probablement plus aigue dans notre laboratoire en raison du milieu de culture utilisé. Ce milieu à base de Earle, contient 1°/₀₀ d'acides aminés sous forme d'hydrolysat de protéines plasmatiques bovines (Travamine-Baxter) et est donc plus pauvre en acides aminés que le TCM 199 de Parker.

Diverses expériences ont montré que le pourcentage en acides aminés est un facteur déterminant pour la filtration du virus. Le tableau en page 267 en est un premier exemple, dans le cas du virus 1342, le pourcentage en acides aminés est le double de celui du virus $MEF_1$ absorbé. Au cours d'une autre expérience (fig. 3) nous avons observé qu'avec un même volume, un pourcentage différent d'acides aminés pouvait entraîner une meilleure filtration du virus. Un même lot de virus est filtré en 3 fractions dans des concentrations différentes d'acides aminés. Lorsque la concentration est de 3°/₀₀, l'absorption est nulle dans la 3 ème fraction, et le virus est entièrement récupéré.

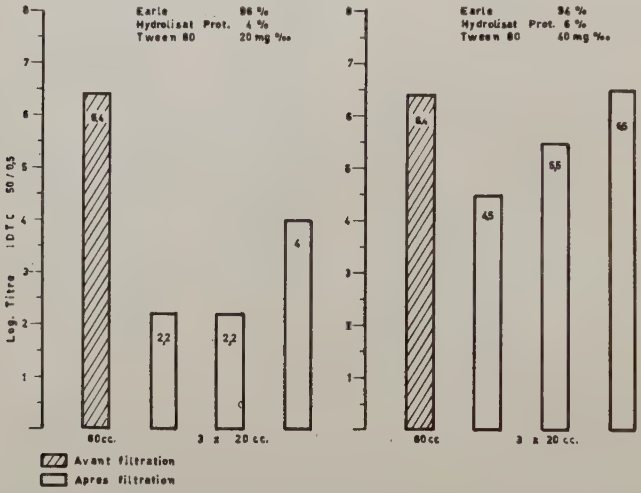

Fig. 3. Filtration sur G 5 (ø 3 cm) en 3 échantillons de 20 cc (Lot 22 $MEF_1$).

D'autre part, la filtration de cultures de virus préparées selon la technique de Maitland, s'accompagne d'une absorption moindre que dans le cas de cultures obtenues sur cellules fixes. La technique de Maitland ou technique de fragments tissulaires en suspension dans le milieu de culture, se caractérise par un pourcentage plus élevé en acides aminés et en protéines étrangères.

Le professeur G. Olin de Stockholm (communicaton personnelle) nous a communiqué avoir observé une absorption lors de la filtration du virus poliomyélitique formolé. Cette absorption est moins importante lorsque le milieu habituel (TCM 199) est enrichi au moyen de glycine ($1,5^{\circ}/_{\circ\circ}$).

## II. Elution du virus.

Au cours d'expériences de filtration où une absorption du virus était observée, nous avons recherché la possibilité de récupérer le virus absorbé sur le filtre Seitz. La filtration de 10 L de virus s'accompagne d'une absorption proche de 90%. La différence d'absorption entre les milieux contenant $1^{\circ}/_{\circ\circ}$ ou $3^{\circ}/_{\circ\circ}$ d'acides aminés est peu importante, la quantité de virus filtré dans cette expérience est en effet à la limite du volume critique de récupération.

Mais un lavage du filtre au moyen d'une solution aqueuse de Tween 80 libère, par élution, 42% du virus absorbé dans le premier cas (milieu à $1^{\circ}/_{\circ\circ}$ d'acides aminés) et 75% dans le second cas (milieu à $3^{\circ}/_{0}$ d'acides aminés). L'élution du second échantillon est donc nettement supérieure (fig. 4). Il est important de noter que l'échantillon de Tween récupérant 35% du virus (217 cc) possède un titre de $10^8$. Cette technique d'élution représente une méthode simple de préparer des échantillons de virus concentré. L'incorporation du Tween 80 dans le milieu de filtration est donc un facteur certain de filtrabilité du virus. Ce facteur ne pose pas de problème puisqu'il se trouve présent dans le TCM 199.

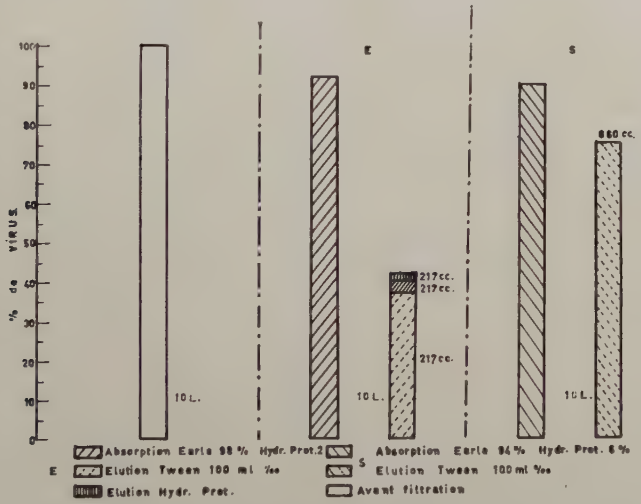

Fig. 4. Absorption et élution avec Filtre Seitz $EKS_1$ (Lot $MEF_1$).

## Conclusions et résumé.

Les travaux expérimentaux réalisés dans notre laboratoire ont démontré la présence d'une absorption lors de la filtration du virus poliomyélitique sur filtres Seitz. Cette absorption est maximale dans les premières fractions filtrées. Lorsque la filtration est effectuée avec un milieu de culture riche en acides aminés (3°/₀₀), contenant du Tween 80 (60 mg °/₀₀) à raison de 250 $cm^3$ par $cm^2$ de surface, la récupération du virus filtré est quasi totale.

Ces conditions de filtration du virus poliomyélitique permettent d'appliquer rigoureusement les mesures de sécurité au cours de la fabrication du vaccin antipoliomyélitique (double filtration sur filtre Seitz $S_1$) sans crainte d'une perte d'efficacité du produit final.

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(Laboratory of Microbiology, Amsterdam, Holland).

# STUDIES ON BACTERIAL ESTERASES

## PART II.—PAPER-ELECTROPHORESIS OF THE ESTERASES OF *PSEUDOMONAS AERUGINOSA* [1])

by

**E. H. M. GREUELL and GONZALO SIERRA**

(Received July 4, 1957).

### 1. INTRODUCTION.

In order to confirm the findings mentioned in Part I, that *Ps. aeruginosa* contains various separate esterases *viz.*, two ali-esterases (triacetinase and tributyrinase) and a lipase, we also tried paper electrophoresis as an independent method. Since with this method it is necessary to deal with very active cell-free preparations, and it is not possible to have in one preparation all three esterases in a very active state (see Part I), we decided to use cell-free extracts (from cells harvested after 5 days of growth) in which lipase and tributyrinase are very active while triacetinase is not present. Therefore, only a separation between lipase and tributyrinase could be attempted. We used horizontal paper-electrophoresis and identified lipase by using p-nitrophenol stearate as chromogenic substrate (HUGGINS and LAPIDES, 1947) and Tween 60 in a modification of the method of SIERRA (1957). Ali-esterase was localized by a pH indicator after reaction of the enzyme with tributyrin. The cell-free enzyme preparation was concentrated by dialysing against a high-polymer solution (WOLVIUS, 1955).

### 2. METHODS.

#### a. Preparation of cell-free enzymes.

The same strain of *Ps. aeruginosa* was cultivated as reported before (Part I). As it was necessary, however, to deal with great volumes of bacterial cultures, cells were harvested first with a

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[1]) Part I: Antonie van Leeuwenhoek **23**, 241, 1957.

Chamberland filter with the technique described by COHEN (1953) and then washed as mentioned (Part I) by centrifuging at 5000 r.p.m. The bacterial mass was ground with glass-powder in a mortar in the same way as in Part I.

### b. **Concentration of the cell-free preparation.**

Since it was observed that by concentrating the preparation by lyophilization, the increment in activity did not keep pace with the reduction of volume, the method of WOLVIUS (1955) was tried for this purpose. This author used a solution of carboxymethyl cellulose (CMC) in distilled water to dialyse a protein rich urine. This method has the advantage that the salt concentration remains about constant when the volume of the cell-free preparation decreases. We used a 15% solution of CMC (AKU, Arnhem, Holland, XT 843), into which was submerged an inverted funnel (4 cm diameter) provided with a cellophane membrane, which contained 5 ml of the enzyme preparation. Dialysis was carried out at 1° C. and after 24 hrs 3.5 ml of the original 5.0 ml solution were left over. The activity of the enzyme preparation, as determined in a Warburg apparatus with tributyrin (see Part I) was 210 $\mu$l $CO_2$/ml/30 min. After dialysis the activity was 720 $\mu$l $CO_2$/ml/30 min. Thus, the activity had increased with a factor 3.4, whereas the volume decreased with a factor 1.4. The explanation of this fact could be that during dialysis some esterase inhibitor is removed.

The concentrated solution remained active for several months when stored at –21° C. The enzyme preparation could also be stored without loss of activity after lyophilization to a dry powder ¹).

### c. **Paper-electrophoresis of the esterases.**

Electrophoresis was carried out in a simple apparatus, which consists mainly of two thick glass-plates provided with glass-frames along the sides. When the two frames were put together, a shallow air-tight room was formed. Five strips of Whatman-1 paper (5 × 40 cm) were pressed horizontally between the glass-frames so that they did not touch the lower glass plate. The polysanite electrode chambers were mounted on each side of the glass-apparatus according to the construction of OOSTERHUIS (1954).

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¹) We thank Dr G. TEN CATE for permitting the use of his lyophilization apparatus.

The strips were moistened by dipping them into the electrolyte (0.025 M solution of Na-veronal-HCl, pH 7.6). The excess of liquid was drained off by pressing the strips on a pad of filter paper and then 0.03 ml of the concentrated enzyme solution was placed at the centre of the strip. 250 volts were applied for 4 hrs, during which the current increased from 8 to 22 mA for five strips. After the moist strips were taken from the chamber, the following procedures were applied.

1. The strips were dried at 105° C. for 10 minutes and then stained with azocarmin B in the usual way to detect the protein-bands (see e.g., KAMPEN and ZONDAG, 1955).

2. In order to detect the ali-esterase on the paper, a replica method was applied. The moist strip was brought in contact with a second strip which was impregnated by dipping it into a 10% solution of tributyrin in acetone and dried before use. The two strips were pressed between glass plates which were closed with vaseline to avoid evaporation. After incubation at 37° C. for 5 hrs, the strip with tributyrin was sprayed with a solution of bromocresol green. The part of the strip which has been in contact with the ali-esterase becomes yellow while the remaining part of the paper is stained blue. Because of the great amount of liberated butyric acid, the influence of the buffer is negligible.

3. Lipase was detected: a) By spraying the moist strip with a 0.2% solution of p-nitrophenol stearate ¹) (HUGGINS and LAPIDES, 1947) in metanol. After incubation between glass-plates at 30° C. for several minutes, yellow bands of p-nitrophenol indicated the position of the lipase. b) By bringing the moist strip in contact with Tween 60 in washed agar which contains small amounts of $CaCl_2$. After incubation at 30° C. microscopical crystals (SIERRA, 1957), mainly observed at the borders of the strip on the zone which has been in contact with the lipase, indicated the position of this enzyme.

### d. **Paper chromatography.**

In order to get information on the adsorption of proteins by the paper, adsorption chromatography of the cell-free preparation was carried out on Whatman-1 paper. The ascending method was used

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¹) This compound was synthesized for us at the Laboratory of Organic Chemistry, University of Amsterdam.

with small strips of paper on which 0.01 ml of the preparation was brought. After chromatography with the buffer solution as liquid phase the strip was stained with azocarmin B or treated for the detection of the esterases.

### 3. RESULTS.

Paper electrophoresis of a cell-free preparation of *Ps. aeruginosa*, which was carried out for 4 hours at 250 volts, showed that the main part of the protein remained at the spot of application. Slight movement towards both poles formed a diffuse band, but 1 cm towards the cathode a clear narrow band could be distinguished. As will be shown, these proteins could not be ascribed to an esterase. Some of the strips were cut longitudinally in two parts, which were used for the different identification methods. Thus lipase was found, with both methods, in a wide band between 0.5 cm towards the cathode and 1.5 cm towards the anode.

The replica method for the detection of ali-esterase showed a yellow band 10 cm towards the anode, whereas the centre of the strip as well as the zone of localization of lipase showed no yellow coloration.

That lipase was not strongly adsorbed on the paper was shown by adsorption chromatography. Lipase was detected only in the region between "$R_F$" 0.7–1.0 (speaking in the terminology of partition chromatography), whereas the greatest part of the proteins remained at the spot of application. A smaller part of the proteins had moved to a Rf-value of 0.5.

As neither lipase nor ali-esterase are significantly adsorbed on the paper, while a great part of the inactive proteins are strongly adsorbed, this opens a simple method for separation and purification of those enzymes. Paper electrophoresis will be used for further purification and for the separation of these two esterases in this laboratory.

## Summary.

From a cell-free enzyme solution of *Ps. aeruginosa* lipase and ali-esterase were separated by means of paper electrophoresis.

## Acknowledgement.

We are greatly indebted to Professor Dr T. Y. KINGMA BOLTJES for his helpful suggestions and stimulating interest.

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(Laboratory of Microbiology, Amsterdam, Holland and Institute "Jaime Ferrán" of Microbiology, Madrid, Spain).

# STUDIES ON BACTERIAL ESTERASES [1]

## PART III. — INFLUENCE OF THE COMPOSITION OF THE CULTURE MEDIA ON THE PRODUCTION OF ALI-ESTERASE AND LIPASE OF *PSEUDOMONAS AERUGINOSA* [2]

by

**GONZALO SIERRA** [3]

(Received July 4, 1957).

### 1. Introduction.

The first recognition of the existence of a difference between a bacterial lipid-hydrolysing enzyme and an ali-esterase was made in the course of the investigations dealing with the specificity of the esterases of *Ps. aeruginosa.* It was noticed that in nutrient broth *Ps. aeruginosa* during its growth produces some different types of esterases. The best known of these esterases are two ali-esterases *viz.*, tributyrinase and triacetinase and a lipase (Sierra, 1957 a; Greuell and Sierra, 1957).

Further experiments have now been carried out to study the influence of the composition of the media on the production of tributyrinase and lipase.

### 2. Culture and methods.

#### a. Culture.

The strain of *Ps. aeruginosa* used in these experiments was the same as that used in our previous investigation (Sierra, 1957 b).

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[1] Part I, II: Antonie van Leeuwenhoek **23**, 241, 273, 1957.

[2] This work was carried out in the Laboratory of Microbiology, Municipal University of Amsterdam, Holland, during the tenure of a grant from the "Consejo Superior de Investigaciones Cientificas", Spain, and finished during an assistantship at the Laboratory of Microbiology.

[3] Address: Instituto "Jaime Ferrán". Joaquin Costa, 32. Madrid. Spain.

### b. Methods.

Propagation of the culture, inoculation, measurement of esterase activity and of the turbidity (to determine the rate of growth) were done in the same manner as reported previously (SIERRA, 1957 b), and also the growth conditions were the same.

### c. Media.

1. Synthetic basal medium. This medium consists of $KNO_3$, 2 g; NaCl, 2 g; $Na_2HPO_4 . 2H_20$, 5 g; $KH_2PO_4$, 1 g; $CaCl_2 . 1 H_2O$, 0.01 g; $MgSO_4 . 7 H_2O$, 0.1 g; a C-source and distilled water, 1000 ml. pH 7.4–7.5.

The calcium chloride solution was sterilized separately and added to the cooled medium. The esters, glycerol and carbohydrates used as C-source were also sterilized separately at 110° C., the esters and glycerol without water, and the carbohydrates and alanine in 10% aqueous solution. To the culture flasks or tubes was added such quantity of each compound as was necessary to get the desired concentration.

2. Nutrient broth. This broth contains Difco Bacto-peptone, 5 g; Liebig beef extract, 3 g; distilled water, 1000 ml. The pH was adjusted to 7.5. Esters to the nutrient broth were added in the same way as in the synthetic medium.

3. Buffered nutrient broth. The same as nutrient broth, but with addition of 5 g $Na_2HPO_4 . 2H_2O$ and 1 g of $KH_2PO_4$ to get a pH of 7.5.

Phosphates, oxalate or fluoride were added to the nutrient broth prior to sterilization. When necessary the pH was always adjusted to 7.5.

## 3. RESULTS.

The influence of the composition of the media on the production of esterases by *Ps. aeruginosa* has been studied in two main types of experiments: (a) by adding esters to the nutrient broth and (b) by adding esters and other substances — as sole C-source — to a mineral medium.

To compare the esterase activity of bacteria grown in different media it is necessary to use always the same amount of cells in the different experiments. In order to standardize the cell suspensions we used a turbidimetric method.

a. **Addition of esters to nutrient broth.**

The purpose of these experiments is to investigate whether the presence of a specific substrate has some influence on the production of esterases by bacteria. We did already show that *Ps. aeruginosa* produces esterases in nutrient broth (SIERRA, 1957 b).

The addition of an ester to nutrient broth usually enhanced the growth of the micro-organism. Striking variations in the capacity of hydrolysing tributyrin by washed cells were observed when cells were grown in the presence of different substrates. Table 1 shows this variation as well as marked changes in the pH values of the medium during the growth due to the action of esterase on the substrate added.

TABLE 1.

Effect of the addition of esters to nutrient broth on ali-esterase activity and growth of *Ps. aeruginosa.*

Different esters were added to the nutrient broth, pH 7.5. Incubation at 37–38° C. for 5 days. Cells were harvested and the washed suspension standardized. Ali-esterase activity was measured on tributyrin.

| | Growth medium: nutrient broth plus: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | none | triacetin 0.5 % | tributyrin % | | | | Tween 20 0.5 % | Olive oil 0.5 % |
| | | | 0.1 | 0.3 | 0.5 | 1.0 | | |
| Growth: bacterial mass (mg D.W./ml) | 0.25 | 1.5 | 0.4 | 0.7 | 2.0 | 5.0 | 3.0 | 5.0 |
| Final pH values | 8.7 | 7.2 | 7.5 | 6.3 | 5.5 | 4.7 | 7.3 | 6.6 |
| Ali-esterase activity ($\mu$l $CO_2$/mg D.W./30 min. 1)) | 670 | 620 | 420 | 0 | 0 | 0 | 600 | 0 |

1) Corrected rate.

Table 1 shows that there is a relation between the pH of the culture medium at the time of harvesting and the capacity of the cells to split tributyrin, and that tributyrinase activity vanishes completely when the medium reaches low pH values. Because of the water solubility of butyric acid the cultures containing tributyrin reach the lowest pH values.

The inhibitory effect of oleate on tributyrinase has already been shown (SIERRA, 1957 b). As lipase activity liberates oleic acid during the growth of the bacteria on olive oil, the absence of the ability of the cells to split tributyrin when grown on olive oil is well explained. Analogous inhibition of the tributyrinase of *Micrococcus pyogenes* var. *aureus* was observed when this bacterium was grown with olive oil. Since *Staphylococcus aureus* also is endowed with lipase activity it is no wonder that the tributyrinase activity of this organism is also diminished by growing it with olive oil.

In order to reduce changes in pH values during the growth, in other series of similar experiments only low concentrations of esters were used in a buffered nutrient broth. The results of these experiments are presented in Table 2, which shows no appreciable variation in the final pH values. Also the capacity of the cells to split tributyrin is virtually the same in all cases.

TABLE 2.

Effect of the addition of esters and glycerol to buffered nutrient broth on the ali-esterase activity of *Ps.aeruginosa*.

Different esters and glycerol were added to buffered nutrient broth (pH 7.5) and incubated for five days at 37–38° C. Cells were harvested and the suspension was standardized. Ali-esterase activity measured on tributyrin.

| | Growth medium: buffered nutrient broth plus: | | | | | | |
|---|---|---|---|---|---|---|---|
| | none | tributyrin % | | | | glycerol 0.5 % | Tween 20 0.5 % |
| | | 0.02 | 0.05 | 0.1 | 0.2 | | |
| Growth: bacterial mass (mg D.W./ml) | 0.25 | 0.25 | 0.25 | 0.25 | 0.70 | 0.30 | 0.25 |
| Final pH values | 8.7 | 8.7 | 8.7 | 8.7 | 8.7 | 8.4 | 8.7 |
| Ali-esterase activity ($\mu$l CO$_2$/mg D.W./ 30 min. 1)) | 25 | 27 | 24 | 25 | 28 | 30 | 26 |

1) Corrected rate.

The capacity of the cell to hydrolyze tributyrin is not increased by the presence of tributyrin, glycerol and Tween 20 in the growth medium. By comparing the results of the experiments of table 1 (column 4 and 8) with those of table 2 (column 5 and 8) we see that the presence of phosphate in the growth medium had a strongly inhibiting effect on the ali-esterase (tributyrinase) activity. From an activity of 650 $\mu$l CO$_2$/mg D.W./30 min in broth it was reduced to 25 $\mu$l CO$_2$/mg D.W./30 min when grown in buffered broth. As cause of this interesting feature some facts will be considered.

As previously reported (SIERRA, 1957 b) cells harvested from broth at the time that lysis of the culture is evident, show a tributyrinase activity higher than before lysis was apparent. Do phosphates influence the lysis or the ali-esterase activity? If so, what is the rôle of the phosphates in this process? Is the inhibiting action of the phosphates due to the simple binding of the metallic ions, which are necessary for the production of esterase or for the lysis of the bacteria?

Addition to nutrient broth of other compounds binding metallic ions such as sodium oxalate and sodium fluoride strongly depressed both ali-esterase and lipase of washed cells growing on these media (see Table 3).

TABLE 3.

Effect of phosphate, fluoride and oxalate on the production of esterase by *Ps. aeruginosa.*

Culture media: nutrient broth and nutrient broth to which before sterilization were added sodium phosphate, sodium fluoride or sodium oxalate. The pH was adjusted to 7.5 if necessary. Temperature of incubation: 37–38° C. Age of the culture five days. Ali-esterase activity was measured with tributyrin. Lipase activity with washed cells which were deprived of ali-esterase activity by exposure for 15 min. to 100° C. (SIERRA, 1957 b) was measured with Tween 80.

| Metallic binding ions added to nutrient broth | Concentration (M) | Esterase activity | |
|---|---|---|---|
| | | Lipase ($\mu$l NaOH 0.1 N/ mg D.W./24 hrs) | Ali-esterase ($\mu$l $CO_2$/mg D.W./30 min.[1]) |
| none | | 150 | 620 |
| phosphate | $3.6 \times 10^{-2}$ | 75 | 30 |
| fluoride | $4.0 \times 10^{-2}$ | 40 | 12 |
| oxalate | $1.5 \times 10^{-2}$ | 0 | 0 |

[1] Corrected rate.

It is noteworthy that there is a close relation between the water solubility of the calcium salt and the degree of inhibition caused by the addition of the metallic binding compounds. Moreover, other influences than removal of alkaline earth cations from the media by oxalate, phosphate and fluoride could be excluded. Already we found that fluoride did not affect the activity of the tributyrinase of *Ps. aeruginosa* (SIERRA, 1957 b). We now also found with manometric experiments that the ali-esterase activity of washed cells suspensions is not affected by an oxalate concentration much higher than the concentrations which completely suppress all ali-esterase activity of growing cultures. Also it is known that in some cases oxalate stabilizes esterase activity (Jansen *et al.*, 1947 a; 1947 b; 1948).

A peculiar influence of the Ca-ions was observed in studies with cholinesterase (van der Meer, 1952): $CaCl_2$ strongly activates cholinesterase but has no influence on pseudocholinesterase and liver esterase. In higher concentrations of $CaCl_2$ the three enzymes are inhibited.

The known facts do not allow a conclusion as to the influence of the Ca-ions in our case. It is probable that Ca-ions themselves are not essential for ali-esterase activity but have some other effect. Curiously enough we found that not always Ca-binding compounds inhibit ali-esterase activity in growing cultures f.i. in cultures of *Bacillus licheniformis* (see Table 4).

TABLE 4.

Effect of metallic ions binding compounds on ali-esterase activity of *Bacillus licheniformis*

The salts were added before sterilization. Temperature of incubation: 30°C., age of the culture three days. Ali-esterase activity was measured on tributyrin.

| Metallic-ions binding compounds added to nutrient broth | Concentration (M) | Ali-esterase activity ($\mu$l $CO_2$/mg D.W./30 min. [1]) |
|---|---|---|
| none | | 60 |
| phosphate | $3.6 \times 10^{-2}$ | 62 |
| fluoride | $4.0 \times 10^{-2}$ | 60 |
| oxalate | $1.5 \times 10^{-2}$ | 59 |

[1] Corrected rate.

As we already noticed, that esterase activity is only manifest after lysis of the bacteria (Sierra, 1957 b), we thought it probable that oxalate, fluoride and phosphates inhibit the esterase activity of growing cultures of *Ps. aeruginosa* by preventing lysis of the bacteria. Therefore we did some experiments to show the influence of these salts upon lysis.

In figure 1 it can be seen that oxalate and phosphate interfere with the lytic phenomenon in the same degree as they inhibit esterase. In nutrient broth cultures containing oxalate no lysis could be observed within 176 hrs.

That the inhibiting effect is mostly due to the binding of Ca-ions was proved as follows: Nutrient broth was freed from cations by passage through a column of „Saucul Brand Base" [1].

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[1] Joseph Grosfield & Sons Ltd., Warrington.

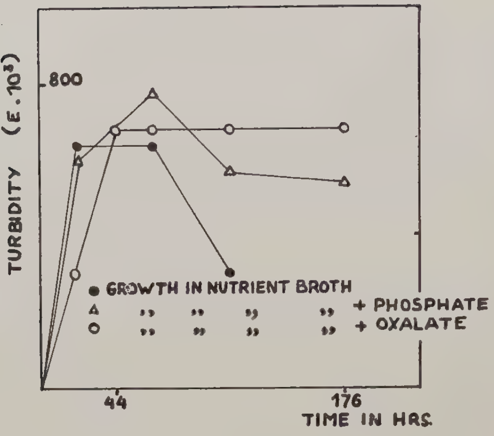

Fig. 1. Effect of oxalate and phosphate added to nutrient broth on the lysis of cultures of *P. aeruginosa* (lysis is shown by a decrease of the turbidity). Temperature: 37–38° C. Final concentration of phosphate and oxalate: $3.6 \times 10^{-2}$ and $1.5 \times 10^{-2}$ M respectively.

The resin was regenerated by floating with an inorganic salt solution and then washed out and treated with hydrochloric acid. Repeated washing with distilled water until $Cl^-$ free. No alkaline earth cations could be detected by precipitation as oxalate. The decalcified broth was neutralized and supplied with 10, 20 and 80 mg% of $CaCl_2 . 1H_2O$, then sterilized and inoculated. After incubation during five days the cultures were harvested and esterase activity was estimated.

TABLE 5.

Effect of Ca-ions on ali-esterase activity of *Ps. aeruginosa*.

Nutrient broth was decalcified by passage through an ion exchanging column, neutralized and supplied with various amounts of $CaCl_2$. 1 $H_2O$. Then sterilized, inoculated and incubated at 37–38°C. for five days. The cells were harvested, the suspension turbidimetrically standardized and ali-esterase measured on tributyrin.

| Concentration of $CaCl_2 . 1 H_2O$ in nutrient medium (mg %) | Ali-esterase activity ($\mu$l $CO_2$/mg D.W./30 min.[1]) |
|---|---|
| none | 0 |
| 10 | 15 |
| 20 | 39 |
| 80 | 40 |

[1] Corrected rate.

Table 5 shows the results of these series of experiments. They first show that there is a complete loss of the esterase activity of the cells after removal of the cations by passage of the nutrient broth through the ion-exchange column and secondly that the esterase activity increases with the concentration of Ca-ions.

The esterase activity is not completely restored by adding Ca-ions. This may be ascribed to the fact that the nutrient broth by flowing through the ion-exchange column, is also deprived of nutrients which stimulate esterase production. Retention of nutrients was well established since the growth of the bacteria in the decalcified nutrient broth was much poorer than in the untreated nutrient broth.

As the esterase activity increases with the degree of lysis and is prevented by substances such as oxalate and phosphate which prevent lysis (Fig. 1), it confirms our former conclusion, that the esterases of *Ps. aeruginosa* are endocellular enzymes.

b. **Addition of esters and other C-sources to a mineral medium.**

A solution or emulsion of the C-source was added to a synthetic medium. It is apparent that each modification causes its particular variation in the pattern of growth. All C-sources were able to initiate growth (see Table 6) although with intervals of different length elapsing between inoculation and visible turbidity (lag phase). Hence growth in each of these media was followed in order to determine the end of the apparently active phase of growth in each culture.

Flask-cultures were harvested one day after reaching the stationary phase of growth. This timing was adopted to gain, as far as possible, similar stages of growth, since already a relation between esterase activity and growth had been found (SIERRA, 1957b).

Cultures grown in synthetic media with different C-sources manometrically show tributyrinase activity except in one case. The absence of tributyrinase activity in cultures grown in media with Tween 80 can be easily explained, because we know that from Tween 80 by lipase action oleic acid is liberated, which inhibits strongly ali-esterase, as has been shown earlier (SIERRA, 1957 b).

The order of suitability as C-source for ali-esterase production is as follows: Tween 20, alanine, glucose, tributyrin, trehalose and glycerol. It must be observed that, although the manometric method gives another sequence than the titrimetric method, in

TABLE 6.

Influence of the composition of the medium on the production of esterases by *Ps. aeruginosa.*

Culture medium: synthetic basal medium plus C-source 0.5% (pH 7.5). Temperature of incubation: 37–38°C. The cultures were harvested one day after the end of the active phase of growth. Lipase activity was estimated with Tween 80 with cells which were exposed for 15 min. to 100° C. Ali-esterase activity was measured with washed cells on tributyrin.

| C-source | Lag phase (days) | End of active phase of growth (days) | Esterase activity | | |
|---|---|---|---|---|---|
| | | | Ali-esterase [1] | | Lipase |
| | | | ($\mu$l $CO_2$/mg D.W./30 min.) | ($\mu$l NaOH 0.1N/ mg D.W./24 hrs) | ($\mu$l NaOH 0.1N/ mg D.W./24 hrs) |
| Tween 80 | 7 | 13 | 0 | 120 | 150 |
| Tween 20 | 4 | 7 | 120 | 340 | 0 |
| Tributyrin | 8 | 13 | 50 | 100 | 3 |
| Glycerol | 7 | 13 | 25 | 440 | 0 |
| Alanine | 2 | 7 | 84 | 230 | 100 |
| Glucose | 3 | 6 | 78 | 150 | 2 |
| Trehalose | 5 | 10 | 48 | 240 | 0 |

[1] Corrected rate.

the whole there is a fairly good agreement between both methods.

Instead of an inductive effect, tributyrin has a depressing effect compared with other C-sources. It should be observed, that cultures in nutrient broth are always much more active (650 $\mu$l $CO_2$/mg D.W./30 min) than those grown in a synthetic medium (120 $\mu$l $CO_2$/mg D.W./30 min,the highest value observed in the synthetic medium with Tween 20).

This situation is similar to that found by CUTCHINS *et al.* (1952) who found a maximal activity for esterase in nutrient broth for *Ps. fluorescens* and attributed this to the fact that nutrient broth provides suitable organic nitrogen and other substances. However, also in the case of *Ps. aeruginosa* the esterase activity is dependent on the degree of lysis in each medium and the lysis was more marked in nutrient broth than in mineral medium.

In contrast to ali-esterase activity, there was only appreciable lipase activity with Tween 80 and alanine. With glucose, trehalose, Tween 20 and tributyrin, lipase activity was so slight, that it could be neglected. Tween 80 as energy source and substrate for the lipase proved to be as expected the most effective substance to produce lipase. More or less to our astonishment alanine also stimulated the production of lipase.

## 3. DISCUSSION.

The influence of Ca-ions upon the lysis of cultures of *Ps. aeruginosa* and the relationship found between esterase activity and lysis are in agreement with previous observations. Esterase activity of *Ps. aeruginosa* seems to be the result of the liberation of the enzyme from the cell by lysis, because: a) the increase of esterase activity corresponds with the increasing degree of lysis of the cultures (SIERRA, 1957 b) and b) it proved to be possible to extract it from crushed bacteria.

The results collected in Table 6 are particularly interesting because bacterial ali-esterase turns out to be present in all media. Nevertheless ali-esterase does not appear to be essential for the synthetic processes of growth (SIERRA, 1957 b). On the other hand, bacterial lipase seems to be an enzyme which is only produced under special circumstances. Adaptive lipases were produced by *Mycobacterium phlei*, *Vibrio tyrosinatica* and various species of the genus *Bacillus* (SIERRA, 1957 a).

The ali-esterase as constitutive enzyme is of more importance for the metabolism of bacteria than the adaptive lipase. However, until the present their function is a matter of conjecture. It will be interesting to know which are the natural substrates of the ali-esterases, so that we can understand the physiological function of these enzymes.

The production of lipase by *Ps. aeruginosa* in nutrient broth may be due to the fact that the presence of lipids in this medium cannot be excluded. Furthermore, according to BEEBE and BUXTON (1905) and HETTCHE (1934) cultures of *Ps. aeruginosa* produce large quantities of lipids in a protein medium free from sugar and fat, and it is therefore possible that the lipids produced in earlier stages of growth may induce the production of lipase in a later stage of growth.

Finally, a noteworthy fact is that Tween 20 as C-source in the mineral medium did not cause lipase formation when measured with Tween 80. This may be an indication of the specificity of the inducer in promoting adaptation. However, this question needs further attention. That in certain cases adaptive enzymes exhibit great specificity towards the substrate which has stimulated their production has been shown by KARSTRÖM (1930) and DUBOS (1940).

## Summary.

The dependence of the production of esterase by *Ps. aeruginosa* on the nutrient medium was studied (a) by supplying esters to nutrient broth and (b) by supplying esters and other C-sources to a mineral medium.

The presence of tributyrin in nutrient broth does not increase the capacity of the cells of splitting tributyrin. Suppression of the esterase activity of the cells was obtained by addition of substances such as oxalate, phosphate, or fluoride which are not toxic for the bacteria and can even stimulate growth. As these compounds do not inhibit directly esterase activity, but prevent lysis of the cells by binding Ca-ions, all these results can be explained by the fact that the esterases of *Ps. aeruginosa* are endocellular enzymes.

Lipase and ali-esterase seem to play a rather different rôle in the metabolism of bacteria. Lipase activity appears to be adaptive, since certain inducers are necessary for its production, while *Ps. aeruginosa* appears to be endowed with ali-esterase activity in all the media studied.

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# BACTERIEN-HAEMAGGLUTINATION

## V. WEITERE VERSUCHE ÜBER HEMMUNG DER COLI-HAEMAGGLUTINATION DURCH CHEMISCHE STOFFE [1]

von

**W. A. COLLIER** [2] und **V. A. H. TIGGELMAN-VAN KRUGTEN**

(Eingegangen am 3. Mai 1957).

Die Haemagglutination durch *E. coli* wird nach den Versuchen von Collier, Sue Tsiew Wong und De Miranda (1955) durch Urin, Normalsera von Huhn und Mensch, durch Organextracte und durch Hühner-Eiklaar gehemmt, ebenso nach Collier und De Miranda (1955) durch einige Kohlehydrate. Da in der Praxis die Hemmung der Virus-Haemagglutination durch specifische Immunsera für die Diagnostik eine wichtige Rolle spielt, ist die Kenntnis unspecifischer hemmenden Stoffe nicht ohne Bedeutung. Wenn auch die Haemagglutination durch *E. coli* nicht ohne Weiteres der Virus-Haemagglutination gleich zu setzen ist, so zeigen sich doch gewisse Parallelen. Aus diesem Grunde wurden die früheren Versuche über unspecifische hemmende Stoffe fortgesetzt.

### Technik.

Die Technik der Haemagglutination und der Hemmungsversuche ist die gleiche wie in den oben erwähnten Arbeiten. Die Kohlehydrate, Aminosäuren, biochemischen Stoffe stammen von ,,General Biochemicals, Chagrin Falls, Ohio'', Albumin und Ovalbumin sind Praeparate der Pharmacopee. Die Pflanzenextracte stammen von frischen Pflanzen, die im Waring-blender in physiologischer Kochsalzlösung zerkleinert wurden. Alle Lösungen wurden vor Gebrauch neutralisiert.

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[1] I, II, III, IV Mitteilung: Antonie van Leeuwenhoek **21**, 113, 124, 133, 303, 1955.

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TABELLE 1.

Hemmung der Haemagglutination von Hühner-und Meerschweinchenblut durch *E. coli* (Stamm 65) mittels Kohlehydrate.

| Hemmendes Kohlehydrat: | Haemagglutination durch das Kohlehydrat: |  | Hühnerblut: Multiplum der haemaggl. Dosis: |  |  | Meerschweinchenblut: Multiplum der haemaggl. Dosis: |  |  |
|---|---|---|---|---|---|---|---|---|
|  | positiv | negativ | 1× | 2× | 4× | 1× | 2× | 4× |
| d(-) Arabinose | 1/20 | 1/40 |  | 0-1/40 | 0 | 0 | 0 | 0 |
| l(+) Arabinose | 1/40Lyse | 1/80 | 0 | 0 | 0 | 0 | 0 | 0 |
| Dextrin | 1/40 | 1/40-1/80 | 1/160 | 0-1/40 | 0 | 0 | 0 | 0 |
| l-Erythrol |  | 1/20 | 0-1/160 | 0-1/40 | 0-1/40 | 0 | 0 | 0 |
| Glucosamin |  | 1/20 | 0 | 0 | 0 |  |  |  |
| d(+) Glucose |  | 1/20 | 1/320-1/640 | 1/80-1/320 | 1/40 | 0-1/40 | 0-1/40 | 0-1/40 |
| β-d-Glucose |  | 1/20 |  | 1/40-1/80 | 0-1/40 | 0 | 0 | 0 |
| Glycogen |  | 1/20 | 0-1/40 | 0 | 0 | 0 | 0 | 0 |
| l-Inositol |  | 1/20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inulin |  | 1/20 |  | 1/80-1/320 | 1/40-1/320 |  | 1/640 | 1/20 |
| β-Lactose | 1/40 | 1/40-1/80 | 0 | 0 | 0 | 0 | 0 | 0 |
| D(+) Lactose |  | 1/20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mannose | 1/20 | 1/20-1/40 |  | bis<br>1/2600000 | bis<br>1/1300000 |  | 1/81920 | 1/20480 |
| α-Methyl-d-Mannosid |  | 1/20 |  | bis<br>1/2600000 | bis<br>1/1300000 |  | 1/163840 | 1/81920 |
| d(+) Melibiose |  | 1/20 |  | 0 | 0 |  | 0 | 0 |
| D(+) Melizitose |  | 1/20 | 1/160 | 1/20-1/80 | 0-1/20 | 1/40 | 0 | 0 |
| d(+) Raffinose |  | 1/20 | 1/40-1/640 | 1/40-1/320 | 0-1/160 | 1/40 | 0 | 0 |
| d(—) Ribose | 1/20 | 1/40 | 0 | 0 | 0 | 1/40 | 0 | 0 |
| D(+) Sucrose (Saccharose) |  | 1/20 | 1/160-1/320 | 1/40-1/80 | 1/20-1/40 | 1/160 | 0-1/40 | 0-1/20 |
| D-Sorbithydrat | 1/40 | 1/40-1/80 | 0 | 0 | 0 |  | 0-1/40 | 0 |
| d(—) Sorbose |  | 1/20 | 1/640 | 1/20-1/640 | 1/20-1/80 |  | 1/640 | 0 |
| d(+) Trehalosehydrat |  | 1/20 | 1/640 | 1/80-1/160 | 1/40-1/80 |  | 1/40 | 1/20 |
| Xylose |  | 1/20 | 0 | 0 | 0 | 0 | 0 | 0 |

TABELLE 2.

Hemmung der Haemagglutination von Hühner- bzw. Meerschweinchenblut durch verschiedene Stämme von *E. coli* mittels Kohlehydrate.

| Zucker: | Stamm von *E. coli*: | Blut von: | Multiplum der agglut. Dosis: | | | |
|---|---|---|---|---|---|---|
| | | | 1× | 2× | 4× | 8× |
| α(—) Erythrol | 4 | Huhn | 0 | 0 | 0 | 0 |
| | 6 | ,, | | 1/160 | 1/40 | 0 |
| | 65 | ,, | 1/160 | 1/40 | 1/20 | 0 |
| | 108 | ,, | 0 | 0 | 0 | 0 |
| d(+) Glucose | 4 | Huhn | 0 | 0 | 0 | 0 |
| | 6 | Huhn | | 1/160 | 1/40 | 1/40 |
| | | Meerschw. | 1/80 | 1/40 | 0 | 0 |
| | 31 | Huhn | 1/320 | 1/80 | 1/20 | 0 |
| | | Meerschw. | 0 | 0 | 0 | 0 |
| | 65 | Huhn a. | 1/320 | 1/320 | 1/40 | 1/40 |
| | | ,, b. | 1/640 | 1/80 | 1/40 | 0 |
| | | Meerschw. | 1/20 | 0 | 0 | |
| | 108 | Huhn | 0 | 0 | 0 | 0 |
| | | Meerschw. | 1/80 | 0 | 0 | 0 |
| d(+) Raffinose | 4 | Huhn | 0 | 0 | 0 | 0 |
| | 6 | ,, | | 1/320 | 1/160 | 1/160 |
| | 65 | ,, | 1/640 | 1/320 | 1/160 | 0 |
| | 108 | ,, | 0 | 0 | 0 | 0 |
| Saccharose | 6 | Meerschw. | 1/80 | 1/40 | 0 | |
| | 31 | Huhn | 1/320 | 1/160 | 1/20 | 0 |
| | | Meerschw. | 0 | 0 | 0 | |
| | 65 | Huhn | 1/320 | 1/80 | 1/40 | 1/20 |
| | | Meerschw. | 1/160 | 1/20 | 0 | |
| | 108 | Meerschw. | 1/320 | 1/20 | 0 | |
| d(+) Trehalose | 4 | Huhn | 1/40 | 0 | 0 | 0 |
| | 6 | ,, | 1/80 | 1/80 | 1/40 | 1/40 |
| | 65 | ,, | 1/640 | 1/160 | 1/40 | 1/40 |
| | 108 | ,, | 0 | 0 | 0 | 0 |

**Hemmung durch Kohlehydrate.**

Die Versuche wurden mit Blut vom Huhn und vom Meerschweinchen durchgeführt und zwar mit dem Stamm *E. coli* 65. Sie sind in Tabelle 1 zusammengefasst. Von den Kohlehydraten agglutinierten 5 die Erythrocyten, bei einem zeigte sich Haemolyse. Sie wurden daher in höherer Verdünnung benutzt. Die Hemmung wurde bei 1-facher, 2-facher und 4-facher agglutinierenden Dosis von *E. coli* untersucht.

Deutlich ergibt es sich, dass die Haemagglutination von Meerschweinchenblut viel schwächer gehemmt wird, als die von Hüh-

## TABELLE 3.

Hemmung der Haemagglutination von Hühner- und Meerschweinchenblut durch *E. coli* (Stamm 65) mittels Eiweiss, Eiweissderivaten, Aminosäuren und einigen anderen physiologischen Stoffen.

| Hemmendes Chemikal: | Haemagglutination durch das Chemikal: | | Hühnerblut Multiplum d. haemaggl. Dosis | | Meerschweinblut Multiplum d. haemaggl. Dosis | |
|---|---|---|---|---|---|---|
| | positiv | negativ | 1× | 2× | 1× | 2× |
| **Eiweisse und Derivate:** | | | | | | |
| Albumin sol. | | 1/100 | 1/25600 | 1/12800 | 1/102400 | 1/25600 |
| Ovalbumin | | 1/100 | 1/25600 | 1/12800 | 1/102400 | 1/12800 |
| Gammaglobulin, Mensch | | 1/250 | 1/2000 | 1/2000 | 0 | 0 |
| Casein sol. | | 1/100 | 0 | 0 | 0 | 0 |
| Pepton | | 1/25 | 1/800 | 1/100-1/200 | 0 | 0 |
| Detoxin (Keratinhydrolysat) | | 1/20 | +++ | +++ | +++ | +++ |
| Chondroitinsulfat | 1/80 | 1/80-1/160 | +++ | +++ | +++ | +++ |
| Desoxyribonucleinsäure | 1/20 | 1/40 | +++ | +++ | 0 | 0 |
| Natriumnucleat | | 1/20 | 1/640 | 1/320 | 0 | 0 |
| Nucleinsäure | | 1/200 | 1/3200 | 1/400 | 0 | 0 |
| **Aminosäuren** | | | | | | |
| dl-α-Alanin | | 1/20 | 0 | 0 | 0 | 0 |
| β-Alanin | 1/40-1/80 | 1/80-1/160 | 0 | 0 | 0 | 0 |
| l(+) Argininmonohydrochlorid | | 1/20 | 0 | 0 | 0 | 0 |
| l-Asparagin | | 1/20 | 0 | 0 | 0 | 0 |
| dl-Asparagin | | 1/20 | 0 | 0 | 0-1/160 | 0 |
| dl-Asparaginsäure | 1/10000-1/25000 | 1/25000-1/50000 | 0 | 0 | 0 | 0 |
| l(—) Cystin | 1/4-1/8 gesätt. | 1/4-1/16 gesätt. | 0 | 0 | 0 | 0 |
| l(+) Cysteinhydrochlorid | 1/20-1/40 | 1/40-1/80 | 0 | 0 | 0 | 0 |
| Glycin | | 1/20 | 0 | 0 | 0 | 0 |
| dl-Histidinhydrochlorid | | 1/20 | 0 | 0 | 0 | 0 |
| dl-Isoleucin | | 1/100 | 0 | 0 | 0 | 0 |
| dl-Leucin | 1/100 | 1/200 | 0 | 0 | 0 | 0 |
| dl-Lysinmonohydrochlorid | | 1/20 | 0 | 0 | 0 | 0 |
| dl-Methionin | | 1/20 | 0 | 0 | 0 | 0 |
| dl-Phenylalanin | | 1/100 | 0 | 0 | 0 | 0 |
| dl-Thyrosin | 1/2 gesätt. | 1/4-1/8 gesätt. | 0 | 0 | 0 | 0 |
| dl-Tryptophan | 1/2 gesätt. | 1/4 gesätt. | 0 | 0 | 0 | 0 |
| dl-Valin | | 1/20 | 0 | 0 | 0 | 0 |
| **Einige andere Stoffe:** | | | | | | |
| Allantoin | 1/200 | 1/400 | 0 | 0 | 0 | 0 |
| Creatin (anhydr ) | | 1/200 | 0 | 0 | 0 | 0 |
| Creatinin | | 1/200 | 0 | 0 | 0 | 0 |
| Uracil | 1/2-1/4 gesätt. | 1/4-1/8 gesätt. | 0 | 0 | 0 | 0 |
| Thiouracil | | 1/4 gesätt. | 0 | 0 | 0 | 0 |
| 5-Methyluracil (Thymin) | | 1/400 | 0 | 0 | 0 | 0 |
| 6-Methyluracil | | 1/200 | 0 | 0 | 0 | 0 |
| 6-Methylthiouracil | | 1/4 gesätt. | 0 | 0 | 0 | 0 |

nerblut. Bei diesem sehen wir deutlichen Einfluss durch d(+) Glucose, Inulin, d(+)Raffinose, d(—)Sorbose und Trehalose, während die hemmende Wirkung von Mannose und α-Methyl-d-

TABELLE 4.

Hemmung der Haemagglutination von Hühner- bzw. Meerschweinchenblut durch verschiedene Stämme von *E. coli* (gewaschene Bacterien) durch Pepton.

| Stamm von *E. coli*: | Blut von: | Multiplum der agglut. Dosis: | | |
|---|---|---|---|---|
| | | 1× | 2× | 4× |
| 4 | Huhn | 1/200 | | |
| | Meerschw. | 0 | 0 | |
| 6 | Huhn a. | | | |
| | b. | 1/200 | 1/200 | |
| | c. | | 1/200 | 1/100 |
| | d. | 1/1600 | 1/400 | 1/200 |
| | e. | 1/800 | 1/800 | 1/400 |
| | Meerschw. | 0 | 0 | 0 |
| 31 | Huhn | 1/50 | 0 | |
| | Meerschw. | 0 | 0 | 0 |
| 76 | Huhn | 0 | 0 | 0 |
| | Meerschw. | 0 | 0 | 0 |
| 65 | Huhn a. | | 1/100 | 1/50 |
| | b. | 1/800 | 1/200 | 1/100 |
| | Meerschw. | 0 | 0 | 0 |
| 82 | Huhn | 0 | | |
| | Meerschw. | 0 | | |
| 108 | Huhn | 0 | 0 | |
| | Meerschw. | 0 | 0 | |

Mannosid ausserordentlich stark ist. Diese beiden Zucker wirken ebenfalls stark bei Meerschweinchenblut.

Die hemmende Wirkung ist nicht gleichmässig, sondern hängt anscheinend von dem verwendeten Blutmuster und vom Alter der Kultur ab. Daher wurden alle Versuche mehrfach durchgeführt. So hemmte beispielsweise d(—)Sorbose in dem einen Versuch in der Endconcentration von 1/20, in anderen bei 1/80 und wieder in anderen bei 1/640.

Weiterhin wurden 5 Zucker auf die hemmende Wirkung auf die haemagglutinierende Fähigkeit von verschiedenen Coli-Stämmen untersucht. Die Versuche sind in Tabelle 2 zusammengestellt. Es zeigen sich hierbei deutliche Unterschiede zwischen der Beeinflussbarkeit der verschiedenen Stämme.

### Hemmung durch Eiweisse, Eiweissderivate, Aminosäuren usw.

Die Versuche wurden mit der 1-fachen und 2-fachen haem-

TABELLE 5.

Hemmung der Haemagglutination von Hühner- bzw. Meerschweinchenblut durch *E. coli* (Stamm 65) durch Proteinderivate.

| Hemmung durch: | Blut von: | Multiplum der agglut. Dosis: | | | |
|---|---|---|---|---|---|
| | | 1× | 2× | 4× | 8× |
| Chondroitinschwefelsäure | Huhn a. | 1/1280 | 1/640 | 1/320 | |
| | b. | 1/320 | 1/1600 | 1/800 | 1/400 |
| | c. | | 1/2560 | 1/1280 | 1/160 |
| | d. | 1/12800 | 1/3200 | 1/1600 | 1/200 |
| | Meerschw. | | 1/3200 | 1/400 | 1/200 |
| Detoxin (Wülfing) | Huhn a. | 1/1600 | 1/400 | 1/200 | 1/100 |
| | b. | | 1/640 | 1/320 | 1/160 |
| | c. | 1/12800 | 1/1600 | 1/400 | 1/200 |
| | Meerschw. | 1/1600 | 1/200 | 0 | |
| Desoxyribonucleinsäure | Huhn a. | | 1/320 | 1/80 | 1/80 |
| | b. | 1/1280 | 1/1280 | 1/640 | 0 |
| | c. | | 1/1280 | 1/640 | 1/320 |
| | Meerschw. | > | > | > | |

agglutinierenden Dosis von *E. coli* 65 ausgeführt und sind in der Tabelle 3 zusammengestellt. Einzelne der Verbindungen zeigen spontane Haemagglutination, besonders stark dl-Asparaginsäure, l(—)Cystin, dl-Tyrosin, dl-Tryptophan und Uracil.

Aus der Tabelle geht hervor, dass Albumin sol., Ovalbumin, Detoxin und Chondroitinsulfat die Coli-Haemagglutination sowohl von Hühnerblut als auch von Meerschweinchenblut sehr stark hemmen, während menschliches Gammaglobulin, Pepton (Bacto), Natriumnucleat und Nucleinsäure nur die Haemagglutination von Hühnerblut beeinflussen. Die anderen untersuchten Stoffe, nämlich die Aminosäuren usw., erwiesen sich als unwirksam. Sie sind aber doch in die Tabelle aufgenommen, da sich in weiteren, unveröffentlichten Versuchen zeigte, dass manche Aminosäuren (z.B.dl-Lysinmonohydrochlorid, dl-Histidinhydrochlorid, l(+)Arginin-monohydrochlorid) die Haemagglulination durch Pflanzenextracte hemmen.

Die hemmende Wirkung von Pepton (Bacto) wurde unter Verwendung verschiedener *E. coli*-Stämme untersucht. Hierbei ergab es sich, dass Stammesunterschiede bestanden, was deutlich aus der Tabelle 4 hervorgeht.

In zahlreichen Versuchen wurde die hemmende Wirkung von Chondroitinschwefelsäure, Detoxin (Keratinhydrolysat) der Firma

Wülfing und Desoxyribonucleinsäure untersucht. In der Tabelle 5 sind einige dieser Versuche zusammengestellt. Es zeigen sich gewisse Unterschiede bei verschiedenen Blutmustern bei Verwendung des gleichen *E. coli*-Stammes. Bemerkenswert ist ferner, dass Chondroitinschwefelsäure und Detoxin die Haemagglutination von Hühnerblut und Meerschweinchenblut hemmten, die Desoxyribonucleinsäure aber bei Meerschweinchenblut unwirksam ist. Ueber die hemmende Wirkung von Detoxin hatten vorher schon COLLIER und TIGGELMAN-VAN KRUGTEN (1955) berichtet. Die Hemmungswirkung der Desoxyribonucleinsäure scheint uns besonders wichtig zu sein.

Früher schon war festgestellt, dass Menschenserum die Haemagglutination von Hühnerblut durch *E. coli* 65 hemmt. In weiteren Versuchen wurde die hemmende Wirkung von 81 Menschenseren gegenüber der Haemagglutination von Hühnerblut und Meerschweinchenblut durch 4 verschiedene *E. coli*-Stämme untersucht. Die Versuche wurden in Tabelle 6 zusammengestellt. Nur durch 14 von den 81 Seren war die Haemagglutination von Meerschweinchenblut geringfügig zu beeinflussen. Bei den Versuchen mit Hühnerblut war stets Hemmung zu sehen, bei den verschiedenen Stämmen aber ungleich kräftig.

Wenn auch die meisten Menschensera die Haemagglutination von Meerschweinchenblut nicht zu hemmen vermochten, so scheinen doch hemmende Stoffe in larvierter Form darin vorhanden zu sein. In Tabelle 7 ist ein Versuch mit einem Menschenserum wiedergegeben. Im nativen Zustande hemmt es nicht die Haemagglutination von Meerschweinchenblut durch die beiden Stämme *E. coli* 6 und *E. coli* 31. Bei Erwärmen tritt aber deutlich die hemmende Wirkung hervor, um bei 20 bzw. 30 Minuten langem Kochen wieder zu verschwinden. Schon früher hatte sich bei Menschen- und Hühnerserum durch Erhitzen eine Steigerung der hemmenden Wirkung gezeigt, sodass an eine thermolabile antagonistische Componente gedacht wurde.

### Hemmungsversuche mit verschiedenen Chemikalien.

Eine Reihe der verschiedensten chemischen Stoffe erwiesen sich in Concentrationen, die selber nicht agglutinierend wirkten, als indifferent gegenüber der Haemagglutination von Hühnerblut durch *E. coli*. Hierher gehören:

Ammoniumbromid, Ammoniumchlorid, Ammoniumsulfat, Caesiumchlorid, Calciumbromid, Calciumchlorid, Calciumgluconat,

TABELLE 6.

Hemmung der Haemagglutination von Hühner- bzw. Meerschweinchenblut durch verschiedene Stämme von *E. coli* durch ein Menschenserum [1]).

| Stamm von *E. coli*: | Blut von: | Multiplum der agglut. Dosis: | | |
|---|---|---|---|---|
| | | 1× | 2× | 4× |
| 4 | Huhn | | 1/320 | 1/320 |
| | Meerschw. | | 0 | 0 |
| 6 | Huhn | 1/320 | 1/80 | |
| | Meerschw. | 0 | 0 | 0 |
| 65 | Huhn | 1/320 | 1/160 | 1/80 |
| | Meerschw. | 0 | 0 | 0 |
| 108 | Huhn | 1/320 | 0 | 0 |
| | Meerschw. | 0 | 0 | 0 |

[1]) Analog reagierten 67 von 81 Menschensera, während bei 14 weiteren eine leichte Hemmung der Meerschweinchenblut-Haemagglutination festzustellen war.

TABELLE 7.

Einfluss des Erwärmens auf die hemmende Wirkung von Menschenserum auf die Haemagglutination von Meerschweinchenblut durch verschiedene Stämme von *E. coli*.

| Stamm von *E. coli*: | Erwärmen des Serums: | Multiplum der aggl. Dosis: | | | |
|---|---|---|---|---|---|
| | | 1× | 2× | 4× | 8× |
| 6 | nativ | | | 0 | 0 |
| | 56°120′ | | | 1/40 | 0 |
| | 70° 30′ | | | 1/40 | 0 |
| | 80° 30′ | | | 1/640 | 0 |
| | 80° 60′ | | | 1/320 | 1/80 |
| | 100° 5′ | | | 1/80 | 0 |
| | 100° 10′ | | | 1/320 | 1/40 |
| | 100° 20′ | | | 1/640 | 1/80 |
| | 100° 30′ | | | 0 | 0 |
| 31 | nativ | | 0 | 0 | |
| | 80° 30′ | | 1/40 | 0 | |
| | 90° 30′ | | 1/40 | 1/40 | |
| | 100° 10′ | | 1/40 | 1/40 | |
| | 100° 20′ | | 0 | 0 | |

Calciumglycerophosphat, Calciumlactat, Calciumsaccharat, Kaliumbromid, Kaliumchlorid, Kaliumjodid, Kaliumsulfat, Natriumbromid, Natriumchlorid, Natriumsulfat, Magnesiumchlorid, Magnesiumsulfat.

Aluminiumsulfat, Eisenchlorid, Eisensulfat, Eisen-ammoniumsulfat, Kupfersulfat, Kupfer-ammoniumsulfat, Nickelsulfat, Nickelammoniumsulfat, Sublimat, Zinkchlorid, Zinksulfat, Zink-ammoniumchlorid.

Aethylalcohol, Methylalcohol, Formalin, Phenol, Glycerin, Glycol, Dimethylglycol, Piperazin, Thiosinamin, Natrium taurocholicum, Ascorbinsäure, Riboflavin, Chlorophyll.

Benzoesäure, Borsäure, Citronensäure, Harnsäure, Milchsäure, Nicotinsäure, Oxalsäure, Trichloressigsäure, Weinsteinsäure. Coffein, Euphyllin, Theobromin.

### Hemmung durch Pflanzenextracte.

Eine Reihe von Pflanzenextracten wurde auf die hemmende Wirkung gegenüber der Agglutination von Hühnerblut durch *E. coli* 65 untersucht. Ausser den nativen Extracten wurde teilweise auch der gleiche Extract nach 10 Minuten langem Erwärmen im Wasserbade auf 100° C. untersucht. Die Versuche sind in der Tabelle 8 zusammengestellt. In ihr findet sich auch die Concentration angegeben, in welcher der Extract gegebenenfalls selber Hühnerblut agglutiniert.

Von insgesamt 213 untersuchten Extracten agglutinierten 101 Hühnerblut, und von diesen waren 80 (79,2%) nicht imstande, die Haemagglutination von Hühnerblut durch *E. coli* 65 zu hemmen. Nur 21 (20,8%) wiesen eine hemmende Wirkung in Concentrationen auf, die geringer waren als jene, wodurch sie selbst Haemagglutination von Hühnerblut verursachten. Bei diesen Extracten sind also sowohl agglutinierende als auch agglutinationshemmende Stoffe vorhanden.

Allerdings gehören hierher auch vier Extracte, die im nativen Zustand keine Hemmungswirkung aufwiesen, vielmehr erst nach Erhitzen wirksam wurden (s.u.). Ausserdem aber agglutinierten bzw. haemolysierten 13 Extracte nur in starken Concentrationen von mindestens 1/80 und nur 4 Extracte agglutinierten bzw. lysierten in einer Verdünnung von mehr als 1/100. Interessant ist in diesem Zusammenhang, dass zu dieser Gruppe 6 von 18 Extracten von Verbenaceen gehörten.

## TABELLE 8.

Hemmung der Haemagglutination von Hühnerblut durch *E. coli* (Stamm 65) mittels Pflanzenextracte.

| Pflanzenextract von: | Agglutin.-Titer des Extractes: | Extr. nativ Multiplum: | | Extr. 10′100° Multiplum: | |
|---|---|---|---|---|---|
| | | 2× | 4× | 2× | 4× |
| **Acanthaceae:** | | | | | |
| *Asystasia gangetica* Blatt | 1/80 | 1/320 | 1/160 | 1/320 | 0 |
| ,, ,, Blume | 0 | 1/1280 | 1/640 | 1/640 | 1/640 |
| *Barleria cristata* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Beloperona guttata* Blatt | 1/320 | 0 | 0 | | |
| ,, ,, Blume | (1/2560) | 0 | 0 | 0 | 0 |
| *Blechum brownei* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Eranthemum tricolor* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Justicia pectoralis* Blatt | 0 | 1/320 | 1/160 | 1/160 | 0 |
| *Megaskepasma erythrochlamys* Blatt | 1/80 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 0 | 0 | 0 | 0 | 0 |
| *Ruellia tuberosa* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Thunbergia alata* Blatt | 0 | 1/320 | 1/320 | 1/320 | 1/160 |
| *Trichanthera gigantea* Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 1/40 | 0 | 0 | 0 | 0 |
| **Amaranthaceae:** | | | | | |
| *Alternanthera* spec. Blatt | 0 | 1/160 | 0 | | |
| **Anacardiaceae:** | | | | | |
| *Mangifera indica* Blatt | 1/80 | 0 | 0 | 0 | 0 |
| **Anonaceae:** | | | | | |
| *Anona muricata* Blatt | 1/320 | 0 | 0 | | |
| **Apocynaceae:** | | | | | |
| *Allamanda cathartica* Blatt | 0 | 1/160 | 0 | 1/160 | 0 |
| ,, ,, Blume | 1/80 Lyse | 1/640 | 0 | 1/320 | 1/320 |
| ,, ,, Samen | 0 | 1/160 | 0 | 1/320 | 1/160 |
| ,, ,, Fruchtfleisch | 0 | 1/320 | 1/160 | 1/320 | 0 |
| *Cryptostegia madagascariensis* Blatt | 0 | 1/320 | 1/320 | 0 | 0 |
| ,, ,, Frucht | 1/40 | 1/320 | 1/320 | 1/320 | 1/160 |
| *Lochnera rosea* Blatt | 0 | 1/160 | 0 | 1/320 | 1/160 |
| ,, ,, Blume | 0 | 0 | 0 | 1/320 | 1/160 |
| *Plumeria alba* Blatt | 0 | 1/320 | 1/320 | 1/320 | 1/160 |
| *Strophanthus* spec. | 0 | 1/320 | 1/160 | 1/320 | 0 |
| *Tabernaemontana coronaria* Knospe | 0 | 1/320 | 1/160 | 1/160 | 1/160 |
| ,, ,, Blatt | 0 | 1/320 | 1/160 | 1/320 | 1/160 |
| **Araceae:** | | | | | |
| *Amorphophallus variabilis* Blatt | 0 | 1/640 | 1/640 | | |
| *Dieffenbachia picta* Blatt | 0 | 1/640 | 0 | | |
| *Montrichardia arborescens* Blatt | 0 | 1/320 | 1/160 | | |
| **Anonaceae:** | | | | | |
| *Anona musicata* | 1/320 | 0 | 0 | | |
| **Bromeliaceae:** | | | | | |
| *Ananas comosus* Blatt | 0 | 1/640 | 1/640 | | |
| **Cactaceae:** | | | | | |
| *Cactus caesius* | 0 | 0 | | | |
| *Cereus hexagonus* | 0 | 0 | | | |
| *Hylocereus lemairei* | 0 | 0 | 0 | | |
| *Nopalea coccinellifera* | 0 | 0 | | | |
| *Rhipsalis cassytha* Stamm | 1/320 Lyse | 0 | 0 | | |
| ,, ,, Samen | 1/80 | 0 | 0 | | |
| **Caprifoliaceae:** | | | | | |
| *Lonicera japonica* Blatt | 1/320 Lyse | 0 | 0 | | |
| *Sambucus canadensis* Blatt | 0 | 1/1280 | 1/640 | | |

| Pflanzenextract von: | Agglutin.-Titer des Extractes: | Extr. nativ Multiplum: |  | Extr. 10′100° Multiplum: |  |
|---|---|---|---|---|---|
|  |  | 2× | 4× | 2× | 4× |
| **Caricaceae:** |  |  |  |  |  |
| *Carica papaja* Blatt | 0 | 1/320 | 1/320 |  |  |
| **Caryophyllaceae:** |  |  |  |  |  |
| *Drymaria cordata* Blatt | 0 | 0 | 0 |  |  |
| **Combretaceae:** |  |  |  |  |  |
| *Combretum rotundifolium* Blatt | 1/2560 | 0 | 0 | 0 | 0 |
| *Laguncularia racemosa* Blatt | 1/1280 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 1/1280 | 0 | 0 | 0 | 0 |
| *Terminalia catappa* Blatt | (1/640) | 0 | 0 | 0 | 0 |
| **Commelinaceae:** |  |  |  |  |  |
| *Commelina nudiflora* Blatt | 1/320 | 0 | 0 |  |  |
| **Compositae:** |  |  |  |  |  |
| *Vernonia cinerea* Blatt | 0 | 1/640 | 1/320 |  |  |
| **Euphorbiaceae:** |  |  |  |  |  |
| *Aleurites moluccana* Blatt | 1/20480 | 0 | 0 | 0 | 0 |
| *Aleurites montana* Blatt | 1/20480 | 0 | 0 | 0 | 0 |
| *Croton hirtus* Blatt | 0 | 1/1280 |  | 1/640 |  |
| ,, ,, Samen | 0 | 1/160 |  | 1/160 |  |
| *Croton lobatus* Blatt | 0 | 1/320 |  | 1/320 |  |
| ,, ,, Samen | 0 | 1/320 |  | 1/160 |  |
| *Euphorbia heterophylla* Blatt | 0 | 1/320 |  | 1/320 |  |
| ,, ,, Blume | 0 | 1/320 |  | 1/640 |  |
| *Hura crepitans* Blatt | 0 | 1/1280 |  | 1/1280 |  |
| *Jatropha podagrica* Blatt | 0 | 1/640 |  | 1/1280 |  |
| **Gramineae:** |  |  |  |  |  |
| *Acroceras macrum* | 0 | 0 | 0 |  |  |
| *Andropogon citratus* | 1/40 | 0 | 0 |  |  |
| *Andropogon nardus* | (1/40) | 0 | 0 |  |  |
| *Andropogon sorghum* | 0 | 1/160 | 0 |  |  |
| *Axonopus affinis* | 0 | 1/160 | 1/160 |  |  |
| *Axonopus compressus* | 0 | 1/320 | 1/160 |  |  |
| *Bambusa multiplex* | 0 | 0 | 0 |  |  |
| *Bambusa vulgaris* | 0 | 1/160 | 0 |  |  |
| *Brachiaria purpurascens* | 0 | 1/320 | 1/160 |  |  |
| *Chloris guyanae* | 0 | 0 | 0 |  |  |
| *Cynodon dactylon* | 0 | 1/160 | 0 |  |  |
| *Dendrocalamus asper* | 0 | 1/640 | 1/320 |  |  |
| *Digitaria decumbens* | 0 | 1/320 | 1/320 |  |  |
| *Digitaria horizontalis* | 0 | 1/320 | 1/160 |  |  |
| *Gigantochloa apus* | 0 | 1/160 | 0 |  |  |
| *Ischaemum aristatum* | 0 | 0 | 0 |  |  |
| *Ischaemum timorense* | 0 | 0 | 0 |  |  |
| *Melinis minutiflora* | 0 | 0 | 0 |  |  |
| *Oryza sativa* | 0 | 1/160 | 1/160 |  |  |
| *Panicum coloratum* var. *makarikariensis* | 1/160 Lyse | 0 | 0 |  |  |
| *Paspalum dilatum* | 0 | 1/320 | 1/160 |  |  |
| *Paspalum notatum* | 0 | 0 | 0 |  |  |
| *Stenotaphrum secundatum* | 0 | 1/320 | 1/160 |  |  |
| *Tripsacum laxum* | 1/40 | 1/640 | 1/160 |  |  |
| *Vetiveria zizanoides* | 0 | 0 | 0 |  |  |
| *Zea mais* | 1/160 | 1/640 | 1/640 |  |  |
| **Labiatae:** |  |  |  |  |  |
| *Orthosiphon grandiflorus* Blatt | 0 | 0 | 0 |  |  |
| ,, ,, Blume | 0 | 0 | 0 |  |  |
| **Leguminosae:** |  |  |  |  |  |
| *Arachis hypogaea* Blatt | 0 | 1/640 | 1/160 | 1/320 | 1/160 |
| ,, ,, Frucht | 0 | 1/640 | 1/320 | 1/1280 | 1/320 |
| *Calliandra brevipes* Blatt | 1/2560 | 0 | 0 | 0 | 0 |
| *Calopogonium mucunoides* Blatt | 0 | 1/640 | 1/320 | 1/640 | 1/320 |
| ,, ,, Schote | 0 | 1/80 | 0 | 1/160 | 0 |

| Pflanzenextract von: | Agglutin.-Titer des Extractes: | Extr. nativ Multiplum: | | Extr. 10′100° Multiplum: | |
|---|---|---|---|---|---|
| | | 2× | 4× | 2× | 4× |
| *Canavalia ensiformis* Blatt | 0 | 1/160 | 1/80 | 1/320 | 1/160 |
| *Cassia fistulata* Blatt | 0 | 0 | 0 | 1/160 | 0 |
| *Cassia fruticosa* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Cassia reticulata* Blatt | 0-1/160 | 0 | 0 | 1/160 | 0 |
| *Cassia spectabilis* Blatt | 1/160 | 0 | 0 | 0 | 0 |
| *Cassia tora* Blatt | 0 | 1/320 | 1/160 | 1/320 | 1/320 |
| *Cynometra cauliflora* Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| *Derris elliptica* Blatt | 1/2560 | 0 | 0 | 0 | 0 |
| *Desmodium asperum* Blatt | 0 | 1/640 | 1/320 | 1/1280 | 1/640 |
| *Enterolobium cyclocarpum* Blatt | 1/20480 | 0 | 0 | 0 | 0 |
| *Erythrina glauca* Blatt | 0 | 1/640 | 0 | 1/320 | 1/160 |
| *Indigofera subulata* Blatt | 1/1280 | 0 | 0 | 0 | 0 |
| „ „ Samen | (1/2560) | 0 | 0 | 0 | 0 |
| *Inga* spec. I Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| *Inga* spec. II Blatt | 1/20480 | 0 | 0 | 0 | 0 |
| *Inga* spec. III Blatt | 1/640 | 0 | 0 | 0 | 0 |
| *Inocarpus edulis* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Mimosa invisa* Blatt | (1/320) | 0 | 0 | 0 | 0 |
| *Myroxylon pereirae* Blatt | 0 | 1/160 | 0 | 0 | 0 |
| *Peltophorum ferrugineum* Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| *Pueraria phaseoloides* Blatt | 0 | 1/160 | 1/80 | 1/320 | 1/320 |
| *Saracca indica* Blatt | 1/5120 | 0 | 0 | | |
| „ „ Samen | 1/1280 | 0 | 0 | | |
| *Tephrosia maxima* Blatt | 1/160 | 0 | 0 | 0 | 0 |
| *Vigna sinensis* Blatt | 0 | 1/320 | 1/320 | 1/320 | 1/160 |
| **Lythraceae:** | | | | | |
| *Lagerstroemia indica* Blatt | 1/80 | 0 | 0 | | |
| **Malpighiaceae:** | | | | | |
| *Golphinia glauca* Blatt | (1/2560) | 0 | 0 | | |
| „ „ Blume | 1/640 | 0 | 0 | | |
| *Malpighia punicifolia* Blatt | 0 | 0 | 0 | | |
| **Malvaceae:** | | | | | |
| *Gossypium barbadense* Blatt | 1/80 | 0 | 0 | | |
| *Hibiscus abelmoschus* Blatt | 1/80 Lyse | 0 | 0 | 0 | 0 |
| *Hibiscus esculentus* Blatt | 0 | 1/320 | 0 | 0 | 0 |
| „ „ Frucht | 0 | 1/1280 | 1/320 | 0 | 0 |
| *Hibiscus rosa sinensis* Blatt | 0 | 0 | 0 | | |
| „ „ „ rote Blume | 0 | 1/320 | 1/320 | 1/320 | 1/320 |
| „ „ „ rosa Blume | 0 | 1/640 | 1/320 | 0 | 0 |
| „ „ „ gelborange Blume | 0 | 1/1280 | 1/640 | 0 | 0 |
| *Hibiscus schizopetalus* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Hibiscus sabdariffa* Blatt | 0 | 0 | 0 | 0 | 0 |
| „ „ Frucht | 1/160 Lyse | 0 | 0 | 0 | 0 |
| *Hibiscus sororius* Blatt | 0 | 1/160 | 0 | 1/160 | 0 |
| *Malachra alceifolia* Blatt | (1/160) | 0 | 0 | 0 | 0 |
| *Malvaviscus arboreus* Blatt | 0 | 0 | 0 | 0 | 0 |
| *Sida glomerata* Blatt | 1/320 | 1/160 | 0 | 0 | 0 |
| „ „ Samen | (1/80) | 0 | 0 | 0 | 0 |
| *Urena lobata* I Blatt | 1/40 | 0 | 0 | 0 | 0 |
| *Urena lobata* II Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| „ „ Samen | 1/2560 | 0 | 0 | 0 | 0 |
| **Musaceae:** | | | | | |
| *Musa paradisea* var. *kapok* Blatt | 0 | 0 | 0 | | |
| **Myrtaceae:** | | | | | |
| *Eucalyptus* spec. Blatt | 1/10240 | 0 | 0 | | |
| **Nyctaginaceae:** | | | | | |
| *Bougainvillea glabra* Blatt | 1/40 | 1/80 | 0 | | |
| *Mirabilis jalapa* Blatt | 1/40 | 0 | 0 | | |
| **Palmae:** | | | | | |
| *Arenga pumata* Blatt | 1/1280 | 0 | 0 | | |

| Pflanzenextract von: | Agglutin.-Titer des Extractes: | Extr. nativ Multiplum: | | Extr. 10′100° Multiplum: | |
|---|---|---|---|---|---|
| | | 2× | 4× | 2× | 4× |
| *Caryota mitis* Blatt | 0 | 1/320 | 0 | | |
| *Cocos nucifera* Blatt | 1/2560 | 0 | 0 | | |
| *Raphis flabelliformis* Blatt | (1/320) Lyse | 0 | 0 | | |
| **Papaveraceae:** | | | | | |
| *Agremone mescicona* Blatt | 0 | 0 | 0 | | |
| ,, ,, Frucht | 1/640 Lyse | 1/640 | 0 | | |
| **Phytolaccaceae:** | | | | | |
| *Microtea debilis* Blatt | 1/40 Lyse | 0 | 0 | | |
| *Peteveria alliacea* Blatt | 0 | 1/320 | | | |
| **Piperaceae:** | | | | | |
| *Piper* spec. Blatt | 0 | 1/80 | 0 | | |
| **Plumbaginaceae:** | | | | | |
| *Plumbago capensis* Blatt | 1/80 | 0 | 0 | | |
| **Polygalaceae:** | | | | | |
| *Securidaca paniculata* Blatt | 0 | 1/160 | 0 | | |
| **Polygonaceae:** | | | | | |
| *Antigonum leptopus* Blatt | 1/320 | 0 | 0 | | |
| *Coccoloba unifera* Blatt | 1/1280 | 0 | 0 | | |
| **Portulacaceae:** | | | | | |
| *Portulaca grandiflora* Blatt | 0 | 1/160 | 0 | | |
| **Rhamnaceae:** | | | | | |
| *Ziziphus jujuba* Blatt | 1/1280 | 0 | 0 | | |
| **Rubiaceae:** | | | | | |
| *Borreria laevis* Blatt | (1/1280) | 0 | 0 | 0 | 0 |
| *Coffea liberica* Blatt | 1/640 | 1/640 | 0 | | |
| *Ixora coccinea* Blatt | 1/2560 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | (1/5120) | 0 | 0 | 0 | 0 |
| ,, ,, Frucht | 1/20480 | 0 | 0 | 0 | 0 |
| *Ixora finlaysoniana* Blatt | 1/10240 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 0 | 0 | 0 | 0 | 0 |
| *Ixora laxiflora* Blatt | 1/5120 | 0 | 0 | 0 | 0 |
| *Ixora lutea* Blatt | 1/2560 | 0 | 0 | 0 | 0 |
| *Ixora macrothyrsa* Blatt | 1/640 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 1/1280 | 0 | 0 | 0 | 0 |
| *Ixora* spec. Blatt | 1/5120 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 1/80 | 1/640 | 1/320 | 1/1280 | 1/640 |
| *Morinda citrifolia* Blatt | 1/80 | 1/5120 | 1/1280 | 1/2560 | 1/1280 |
| *Oldenlandia herbacea* Blatt | 0 | 1/640 | 1/160 | 1/1280 | 1/320 |
| *Rondeletia odorata* Blatt | 1/5120 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 1/10240 | 0 | 0 | 0 | 0 |
| **Rutaceae:** | | | | | |
| *Murraya exotica* Blatt | 0 | 1/320 | 0 | | |
| **Sapindaceae:** | | | | | |
| *Nephelium litchi* Blatt | 1/1280 | 0 | 0 | | |
| **Sapotaceae:** | | | | | |
| *Achras sapota* Blatt | 1/5120 | 0 | 0 | | |
| **Scrophulariaceae:** | | | | | |
| *Angelonia salicariaefolia* Blatt | (1/40) | 0 | 0 | | |
| **Simarubiaceae:** | | | | | |
| *Quassia amara* Blatt | 1/2560 | 0 | 0 | | |
| **Solanaceae:** | | | | | |
| *Solanum macranthum* Blatt | 0 | 1/80 | 0 | | |
| **Sterculiaceae:** | | | | | |
| *Guazuma ulmifolia* Blatt | 1/40 | 1/320 | | | |
| *Theobroma cacao* Blatt | 1/1280 | 0 | 0 | | |
| **Tiliaceae:** | | | | | |
| *Corchorus capsularis* Blatt | 0 | 0 | 0 | | |

| Pflanzenextract von: | Agglutin.-Titer des Extractes: | Extr. nativ Multiplum: 2× | 4× | Extr. 10′100° Multiplum: 2× | 4× |
|---|---|---|---|---|---|
| **Verbenaceae:** | | | | | |
| *Clerodendron fragrans* Blatt | 1/40 | 0 | 0 | 1/640 | 0 |
| *Clerodendron speciosissimum* Blatt | 0 | 1/640 | 1/320 | 1/640 | 1/320 |
| ,, ,, Blume | 0 | 1/320 | 0 | 1/640 | 1/320 |
| *Duranta erecta* Blatt | 1/320 Lyse | 0 | 0 | 1/640 | 0 |
| ,, ,, Blume | 1/160 Lyse | 0 | 0 | 0 | 0 |
| ,, ,, Beere | 1/160 Lyse | 1/2560 | 0 | 1/1280 | 0 |
| *Cytharexylum spinosum* Blatt | 0 | 1/640 | 1/320 | 1/640 | 1/320 |
| *Holmskioldia sanguinea* Blatt | 0 | 1/640 | 0 | 1/320 | 1/320 |
| ,, ,, Blume | 0 | 1/640 | 1/320 | 1/320 | 1/160 |
| *Lantana camara* Blatt | 0 | 0 | 0 | 0 | 0 |
| ,, ,, Blume | 0 | 0 | 0 | 0 | 0 |
| ,, ,, Frucht | 0 | 0 | 0 | 0 | 0 |
| *Petraea arborea* Blatt | 0 | 0 | 0 | 1/320 | 0 |
| *Stachytarpheta cayennensis* Blatt | 1/320 Lyse | 0 | 0 | 1/640 | 0 |
| ,, ,, Blume | 0 | 0 | 0 | 0 | 0 |
| *Stachytarpheta jamaicensis* Blatt | (1/160) Lyse | 0 | 0 | 1/160 | 0 |
| ,, ,, Blume | (1/80) Lyse | 1/160 | 0 | 1/160 | 0 |
| *Tectona grandis* Blatt | 1/40 | 0 | 0 | 0 | 0 |
| **Zingiberaceae:** | | | | | |
| *Aframomum melegueta* Blatt | 1/40 | 1/320 | 0 | | |
| *Curcuma xanthorrhiza* Wurzel | 1/160 Lyse | 0 | 0 | | |
| *Renealmia exaltata* Blatt | 1/320 Lyse | 0 | 0 | | |
| **Farrenpflanzen:** | | | | | |
| *Adianthum* spec. | (1/2560) | 0 | 0 | | |
| *Asplenium* spec. | 1/80 | 1/160 | 0 | | |
| *Lygodium* spec. | 1/1280 | 0 | 0 | | |
| *Marsilia* spec. | 0 | 1/640 | 1/640 | | |
| *Nephrolepis* spec. | 1/40 | 1/5120 | 1/640 | | |
| *Pteris* spec. | 1/80 Lyse | 1/1280 | 1/320 | | |
| *Salirinia natans* | 0 | 0 | 0 | | |

Diesen Ergebnissen steht gegenüber, dass von 112 Hühnerblut nicht agglutinierenden Extracten 75 (67,0%) die Coli-Haemagglutination hemmten, während 37 (33,0%) keinen Einfluss ausübten. Im Ganzen fanden sich unter den 213 Pflanzenextracten 37 (17,4%), die weder agglutinierten noch hemmten.

Bei 118 Extracten wurde untersucht, ob das Erwärmen (10′100°) Einfluss auf die hemmende bzw. nicht-hemmende Wirkung hat.

Hierbei zeigte sich, dass in 7 Fällen die hemmende Wirkung des Extractes auf die Coli-Haemagglutination aufgehoben wurde. Dies war der Fall bei dem Extract von Blättern von *Cryptostega madagascariensis* (während der Extract aus der Frucht unbeeinflusst blieb), bei Extract aus Blättern von *Myroxylon pereirae*, von rosa Blumen und von gelborangen Blumen von *Hibiscus rosa sinensis* (während der Extract aus roten Blumen positiv blieb), von Blatt und von Frucht von *Hibiscus esculenta* und beim Extract von Blättern von *Sida glomerata* (während der Extract aus dem Samen überhaupt nicht hemmte).

Weiterhin zeigte es sich aber, dass in 8 Fällen der native Extract keine hemmende Wirkung aufwies, wohl aber nach dem Erhitzen. Hierbei ging es um die Extracte von Blumen von *Lochnera rosea* (während der Extract aus Blättern auch nativ hemmte), von Blättern von *Cassia fistula*, *Cassia reticulata*, *Clerodendron fragrans*, von Blättern von *Duranta erecta* (während Extract aus den Blumen weder nativ noch erhitzt und Extract aus der Beere sowohl nativ, als auch erhitzt hemmten), von Blättern von *Petraea arborea* und schliesslich von Blättern van *Stachytarpheta cayennensis* (während der Extract aus der Blume negativ blieb) und *Stachytarpheta jamaicensis* (während der Extract von den Blumen stets positiv war).

Diese Beeinflussung durch Erhitzen zeigt deutlich, dass auch in Pflanzenextracten antagonistische Factoren gegen hemmende Stoffe vorkommen können, analog wie solche bei Seren nachweisbar sind.

Besonderes Interesse hat wegen ihrer gruppenspecifischen Wirkung die Limabohne. Extract hiervon wurde gegen die Haemagglutination von Hühner- und Meerschweinchenblut durch 4 verschiedene Stämme von *E. coli* untersucht. Die Ergebnisse finden sich in Tabelle 9 zusammengestellt. Limabohnenextract hemmte in jedem Falle deutlich, sowohl die Hühner- als auch die Meerschweinchenblut-Haemagglutination.

TABELLE 9.

Hemmung der Haemagglutination von Hühner- bzw. Meerschweinchenblut durch verschiedene Stämme von *E. coli* mittels Extract von Limabohnen.

| Stamm von *E. coli*: | Blut von: | Multiplum der agglut. Dosis: | | | |
|---|---|---|---|---|---|
| | | 1× | 2× | 4× | 8× |
| 6 | Huhn | 1/1280 | 1/1280 | 1/1280 | 1/640 |
| | Meerschw. | | 1/1280 | 1/1280 | 1/640 |
| 65 | Huhn | 1/2560 | 1/1280 | 1/640 | 1/320 |
| | Meerschw. | | | | |
| | a. | 1/640 | 1/320 | 1/160 | 1/80 |
| | b. | 1/1280 | 1/640 | 1/320 | |
| 108 | Meerschw. | | | | |
| | a. | 1/1280 | 1/1280 | 1/640 | |
| | b. | 1/5120 | 1/2560 | | |
| 110 | Meerschw. | 1/2560 | 1/320 | 1/80 | 0 |

## Zusammenfassung.

Die *E. coli*-Haemagglutination wird durch verschiedene Kohlehydrate deutlich gehemmt, besonders stark durch Mannose und α-Methyl-d-Mannosid. Die Haemagglutination von Hühnerblut wird stärker gehemmt als die von Meerschweinchenblut.

Eiweisse und hochmolekulare Abbauproducte können starke Hemmungswirkung aufweisen, Aminosäuren aber nicht. Die hemmende Wirkung ist abhängig von den verwendeten Stämmen von *E. coli*.

Menschenserum hemmt gut die Hühnerblut-Agglutination, wenig oder garnicht die Meerschweinchenblut-Agglutination. Dies ist möglicherweise auf thermostabile Antagonisten zurückzuführen.

Von 101 Pflanzenextracten, die Hühnerblut selber agglutinierten, hemmten 21 die Coli-Haemagglutination von Hühnerblut, von 112 Hühnerblut nicht agglutinierenden Extracten hemmten 75. Auch in einigen Pflanzenextracten scheinen thermolabile Antagonisten gegen die hemmenden Stoffe vorzukommen.

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# THE UTILISATION OF THIOCYANATE AND NITRATE BY THIOBACILLI

by

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BEIJERINCK (1904) was the first investigator to record the microbiological decomposition of thiocyanate. In his classical studies on the metabolism of *Thiobacillus thioparus* and *T. denitrificans* he described the degradation of this compound with profuse separation of sulphur when 0.25% ammonium thiocyanate replaced thiosulphate in the enrichment medium for these organisms.

Thirty years later HAPPOLD and KEY (1937) in their studies on the bacterial purification of gas liquors reinvestigated the bacterial oxidation of ammonium thiocyanate. They established the fact that sewage purification systems could be adapted to bring about the breakdown of this compound. From an adapted sludge they isolated on nutrient agar a Gram-negative, pigmented, rod-shaped organism which in pure culture utilised ammonium thiocyanate, and which they named *Bacterium thiocyanoxidans*. HAPPOLD, JOHNSTONE and ROGERS (1952) and HAPPOLD, JOHNSTONE, ROGERS and YOUATT (1954) studied this organism further. They showed that the original cultures were in all likelihood mixed and succeeded in obtaining in pure culture a Gram-negative, motile, obligate autotrophic rod which quantitatively converted thiocyanate to sulphate and ammonia while obtaining its carbon requirements from carbon dioxide. Energy for growth could also be supplied by the oxidation of sulphur, sulphides and thiosulphate. In accordance with BEIJERINCK'S original observations they classified the organism as a *Thiobacillus* species and proposed the species name *T. thiocyanoxidans*.

YOUATT (1954) studied the metabolism of this organism in considerable detail. She suggested that thiocyanate was first hydrolysed enzymatically to cyanate and sulphide. The latter was oxidised to sulphate whilst the former was further hydrolysed to ammonia and carbon dioxide.

In 1955 VAN DER WALT and DE KRUYFF first obtained evidence of the oxidation of thiocyanate under anaerobic conditions with nitrate as oxidant. Not only did it appear possible to obtain enrichment cultures of denitrifying *Thiobacillus* species with thiocyanate, but it was also shown that pure cultures of *T. denitrificans* readily utilised thiocyanate as sole source of oxidisable sulphur with nitrate as oxidant.

The metabolism of nitrate by *Thiobacilli* has been investigated in detail only in the case of *T. denitrificans*. BEIJERINCK regarded *T. thioparus* as incapable of denitrification. This view was supported by BAALSRUD and BAALSRUD (1954) who stated that it was unable to reduce nitrate or nitrite. HAPPOLD, JOHNSTONE, ROGERS and YOUATT mentioned that *T. thiocyanoxidans* could effect some reduction of nitrate to nitrite but they did not study the phenomenon in detail. BAALSRUD and BAALSRUD made an extensive study of the reduction of nitrate by *T. denitrificans*. They obtained evidence that, in the presence of thiosulphate, the reduction proceeded via nitrite, nitric oxide and nitrous oxide, the end product being nitrogen. Nitrite only accumulated in the medium if there was insufficient thiosulphate for complete reduction. VERHOEVEN (1956) confirmed that *T. denitrificans* could reduce nitrite to nitric oxide which in turn could be reduced to nitrogen. Nitric oxide only accumulated under physiologically adverse conditions.

In view of the findings of VAN DER WALT and DE KRUYFF, as well as those of BEIJERINCK, the present study was undertaken to determine to what extent the ability to utilise thiocyanate was found amongst different species of *Thiobacilli* and whether this property was a valid criterion for species differentiation. When it was found that in addition to *T. thiocyanoxidans*, *T. thioparus* and *T. denitrificans* could also utilise thiocyanate as sole sulphur source, a study of the reduction of nitrate under anaerobic conditions in the presence of thiocyanate or thiosulphate was undertaken to investigate the relationships between the three species.

## METHODS.

### Cultures.

The following cultures were used:

*Thiobacillus thioparus*: strain NCIB 8370 from the Chemical Research Laboratory, England; locally isolated strains A2–1 and Kl–1.

*Thiobacillus denitrificans*: strain vN, kindly made available by Professor C. B. VAN NIEL; locally isolated strain A3–1.

*Thiobacillus thiooxidans*: strain NCIB 8343.

*Thiobacillus thiocyanoxidans*: strain NCIB 8572.

### Media.

**Thiosulphate-agar medium A:** $Na_2S_2O_3.5H_2O$ 0.5 g, $K_2HPO_4$ 0.4 g, $KH_2PO_4$ 0.4 g, $MgSO_4 . 7H_2O$ 0.01 g, $CaCl_2 . H_2O$ 0.115 g, $MnSO_4 . 4H_2O$ 0.025 g, $FeCl_3 . 6H_2O$ 0.002 g, $(NH_4)_2SO_4$ 0.01 g, washed agar 2 g, tap water 100 ml; pH 7.3.

**Thiosulphate-nitrate agar medium B:** Double strength medium F 100 ml, 4% washed agar 100 ml; pH 6.7.

**Thiocyanate medium C:** KCNS 0.02 g, $Na_2HPO_4$ 0.10 g, $KH_2PO_4$ 0.06 g, $MgSO_4 . 7H_2O$ 0.002 g, $FeCl_3 . 6H_2O$ 0.002 g, tap water 100 ml; pH 6.7.

**Thiocyanate medium D:** KCNS 0.02 g, $KH_2PO_4$ 0.3 g, $MgSO_4 . 7H_2O$ 0.05 g, $CaCl_2 . H_2O$ 0.03 g, $FeCl_3 . 6 H_2O$ 0.002 g, $(NH_4)_2SO_4$ 0.02 g, dist. water 100 ml; pH 4.8.

**Thiocyanate-nitrate medium E:** $NH_4CNS$ 0.02 g, $KNO_3$ 0.10 g, $K_2HPO_4$ 0.075 g, $KH_2PO_4$ 0.10 g, $NH_4Cl$ 0.10 g, $MgCl_2 . 6H_2O$ 0.05 g, $Na_2CO_3$ 0.05 g, $FeCl_3 . 6H_2O$ 0.002 g, tap water 100 ml; pH 6.7.

**Thiosulphate-nitrate medium F:** $Na_2S_2O_3 . 5H_2O$ 0.5 g, $KNO_3$ 0.5 g, $K_2HPO_4$ 0.075 g, $KH_2PO_4$ 0.10 g, $NH_4Cl$ 0.10 g, $MgCl_2 . 6H_2O$ 0.05 g, $Na_2CO_3$ 0.05 g, $FeCl_3 . 6H_2O$ 0.002 g, tap water 100 ml; pH 6.7.

### Cultivation of Organisms.

For inoculation *T. thioparus* and *T. thiocyanoxidans* were cultured aerobically on plates containing medium A; *T. denitrificans* was cultured anaerobically on medium B.

For the aerobic experiments, single colonies were selected from the plates and inoculated into 75 ml of medium C or D in 250 ml

Erlenmeyer flasks. The organisms were grown for 3 weeks at 30° C.

For the anaerobic experiments, single colonies were inoculated into 75 ml of medium E or F contained in the apparatus shown in Fig. 1. This apparatus was designed to maintain anaerobic conditions and to permit the collection of the gaseous metabolic products.

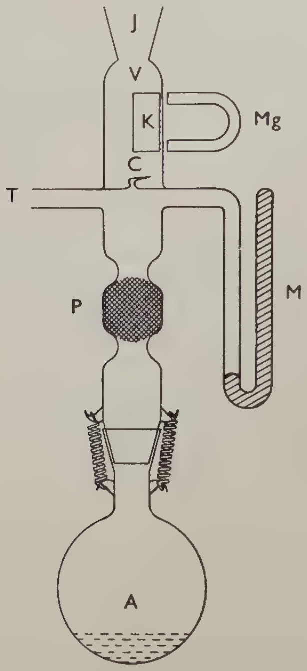

Fig. 1. Apparatus for anaerobic cultivation of Thiobacilli for quantitative experiments.

The apparatus consisted of the culture flask A, a 100 ml round-bottomed flask with a ground-glass joint to which could be fitted a superstructure in the form of a tube carrying a cotton plug P, a manometer M, a side tube T and a fine sealed capillary C, projecting into a chamber V which was fitted with a ground-glass joint J. The ground-glass joints were greased with a silicone grease and the apparatus was sterilised by dry heating for 1 hour at 180° C. After introduction of the medium into A and inoculation, the superstructure was replaced and the apparatus evacuated through the side arm T until the medium commenced boiling under the reduced pressure. The evacuation was continued for ½ hour to remove all traces of oxygen. The side arm T was then sealed off. The degree of anaerobiosis so obtained was sufficient for Fildes leuco-methylene blue-glucose indicator to remain colourless for at least 2 weeks. After cultivation of the organisms for circa 3 weeks at 30° C., a soft iron anchor K was carefully placed in V. V was then connected via J to a gas sampling tube and manometer. After evacuation of the system, C was broken with the aid of K and a magnet Mg. The pressure was noted and the gas drawn off for quantitative analysis.

### Analytical Methods.

The media from the experiments and the uninoculated controls were analysed by the following methods:

**Thiocyanate** was determined colorimetrically with silver nitrate (SNELL and SNELL, 1948).

**Sulphate** was determined gravimetrically as $BaSO_4$ (VOGEL, 1951).

**Elementary sulphur** was determined as sulphate after oxidation with bromine (SCOTT, 1946).

**Ammonia** was determined by alkaline distillation into 0.2 N HCl and back titration with 0.2 N NaOH.

**Nitrite** was determined colorimetrically with sulphanilic acid and $\alpha$-naphthylamine after oxidisable sulphur compounds had been removed by precipitation with silver nitrate (SNELL and SNELL, 1948; PIERSON, 1954).

**Nitrate**: Nitrogen present as nitrate, nitrite and ammonia was determined as ammonia after reduction with Devarda alloy, thiocyanate having first been removed by precipitation with silver acetate (VOGEL, 1951). Nitrate N was estimated by difference between the value so obtained and the sum of nitrite N and ammonia N.

**Gaseous nitrogen compounds.** The gaseous products of denitrification were analysed in the mass spectrograph.

## RESULTS.

### Aerobic dissimilation of thiocyanate.

Since BEIJERINCK had obtained thiocyanate decomposition in enrichment cultures for *T. thioparus* from surface water with addition of ammonium thiocyanate, it was attempted first to cultivate this organism in the simple inorganic medium prescribed by HAPPOLD *et al.* for the cultivation of *T. thiocyanoxidans*. Although cultures remained viable in this medium, no proliferation to any marked degree nor decomposition of thiocyanate occurred. Efforts to adapt cultures to thiocyanate by repeated transfer from a medium containing thiosulphate and thiocyanate to media containing progressively more thiocyanate and less thiosulphate also failed when thiosulphate was finally omitted.

Vishniac (1952) had, however, observed that *T. thioparus* only developed readily in thiosulphate media if at least 0.02 g per litre ferric chloride had been added. After this quantity of ferric chloride, together with traces of magnesium sulphate, had been added to Happold's basal medium, it was found to support excellent growth of *T. thioparus*, even after innumerable transfers. The modified medium (medium C) also supported prolific growth of *T. denitrificans* and *T. thiocyanoxidans*. Quantitative experiments (Table 1) showed that the three species metabolised thiocyanate with equal facility converting it practically quantitatively to sulphur and sulphate.

TABLE 1.

Aerobic metabolism of thiocyanate.

(21 days cultivation in medium C at 30°C.).

| Species | pH | | Thiocyanate utilised | Sulphur formed | Sulphate formed |
|---|---|---|---|---|---|
| | before | after | ($\mu$M per 75 ml medium) | | |
| *T. denitrificans*<br>(Strain A3.1) | 7.25 | 7.12 | 213 | 28 | 180 |
| *T. thioparus*<br>(N.C.I.B. 8370) | 7.41 | 7.28 | 228 | 16 | 192 |
| *T. thiocyanoxidans*<br>(N.C.I.B. 8572) | 7.18 | 7.08 | 194 | 10 | 169 |

*T. thiooxidans*, on the other hand, failed to grow on this medium. This was not unexpected since the pH was higher than tolerated by this organism. Accordingly, it was attempted to cultivate *T. thiooxidans* in the medium prescribed by Starkey (1925) in which the elementary sulphur was replaced by potassium thiocyanate and the pH adjusted to 4.8 (medium D). The results, however, were again negative.

### Anaerobic metabolism of thiocyanate with nitrate as oxidant.

The oxidation of thiocyanate by *T. denitrificans* under anaerobic conditions with nitrate as oxidant was demonstrated by VAN DER WALT and DE KRUYFF (1955). Further work showed that *T. thiocyanoxidans* and *T. thioparus* could also grow anaerobically on a thiocyanate medium containing nitrate (medium E). The finding that *T. thioparus* could grow under these conditions was unexpected since this organism has always been regarded as strictly aerobic. The experimental conditions, however, were such as to exclude all possibility of molecular oxygen acting as hydrogen acceptor in the oxidation of the thiocyanate.

Quantitative studies showed that the mode of utilisation of nitrate by *T. thioparus* and *T. thiocyanoxidans* differed from that of *T. denitrificans*. Typical results are presented in Table 2.

TABLE 2.

Anaerobic metabolism of thiocyanate. (21–25 days growth at 30° C. in medium E; all quantities given in $\mu$M per 75 ml medium).

| Species | pH | | Metabolites consumed | | Metabolites formed | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | before | after | Thiocyanate | Nitrate | Sulphur | Sulphate | Ammonia | Nitrite | Nitrogen |
| *T. denitrificans* (Strain vN) | 7.41 | 7.12 | 198 | 180 | 12 | 188 | 24 | 1 | 480 |
| *T. thioparus* (N.C.I.B. 8370) | 7.83 | 7.30 | 229 | 164 | 20 | 206 | 260 | 155 | 0 |
| *T. thiocyanoxidans* (N.C.I.B. 8572) | 7.38 | 7.00 | 144 | 37 | 17 | 66 | 2 | 39 | 0 |

After 21 days *T. denitrificans* had converted the thiocyanate in the medium practically quantitatively to sulphur and sulphate, with the concomitant reduction of the nitrate to nitrogen. Only a trace of nitrite was found in the medium at the end of the experiment. *T. thioparus* and *T. thiocyanoxidans* also oxidised the thiocyanate to sulphur and sulphate, but reduced the nitrate only as far as nitrite. They did not produce any measurable quantity of gas.

### Anaerobic metabolism of thiosulphate with nitrate as oxidant.

*T. thioparus* and *T. thiocyanoxidans* were also found to be capable of anaerobic growth in a medium containing thiosulphate and nitrate (medium F), which is essentially the same as that used by BAALSRUD and BAALSRUD for the cultivation of *T. denitrificans*.

In this medium *T. thioparus* and *T. thiocyanoxidans* again differed from *T. denitrificans* with respect to the stage to which they were capable of reducing nitrate (Table 3). As expected, it was found that after 20 days *T. denitrificans* had reduced the nitrate practically quantitatively to nitrogen and that only a trace of nitrite was present in the medium. In the case of *T.thiocyanoxidans*, more than 80% of the nitrate reduced was found as nitrite. A small amount of nitrogen was also produced. It is noteworthy that in one experiment the amount of nitrite produced was extremely high, the concentration at the end of the experiment being 4.2 mM per 75 ml medium, corresponding to 2.9 g $NaNO_2$ per litre. In other experiments, however, the concentrate of nitrite produced did not exceed 0.2 mM per 75 ml medium. *T. thioparus* produced no measurable quantity of gas. Nitrite was the principal reduction product found in the medium at the end of the experiment. It accounted, however, for only 30–50% of the nitrate reduced. In some experiments the amount of ammonium ion present increased whereas in other experiments it decreased. In no case could all the nitrate reduced be accounted for. Tests for hydroxylamine, hydrazine and protein nitrogen were either negative or accounted for only traces of nitrogen. Losses of nitrogen were also reported by VERHOEVEN (1952) in the case of the reduction of nitrate by aerobic sporeforming bacteria. These were shown to be due to the presence of dissolved nitrous oxide in the medium which escaped analysis. This may also have occurred in our experiments. The absence of any gas formation, even though the pressure in the culture flask was only 30–40 mm mercury, makes it doubtful, however, if this could account for all the deficit.

Owing to the difficulty of estimating thiosulphate in the presence of nitrite, it was not possible to follow the utilisation of thiosulphate in the above experiments. It could be shown, however, that the growth of all three species led to the accumulation of sulphate and sulphur in the medium.

## DISCUSSION.

The present work has shown that, in addition to HAPPOLD'S *T. thiocyanoxidans*, several strains of *T. thioparus* and *T. denitrificans* are able to utilise thiocyanate as sole source of sulphur, both

TABLE 3.

Anaerobic metabolism of thiosulphate.

(Growth on medium F at 30° C.; all quantities given in $\mu$M per 75 ml medium).

| Species | Growth period (days) | pH | | Nitrate utilised | Ammonia formed (+) or utilised (—) | Metabolites formed | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | before | after | | | Nitrite | Nitrogen | Sulphur | Sulphate |
| *T. denitrificans* (Strain A$_3$) | 20 | 7.37 | 7.07 | 444 | — 28 | 8 | 410 | 79 | 274 |
| *T. thioparus* NCIB 8370 | 24 | 7.27 | 7.09 | 661 | + 117 | 237 | 0 | 112 | 810 |
| *T. thioparus* NCIB 8370 | 28 | 7.01 | 7.07 | 650 | — 98 | 177 | 0 | 105 | 399 |
| *T. thiocyanoxidans* NCIB 8572 | 19 | 7.81 | 7.26 | 3,920 | — 52 | 4,184 | 1) | 21 | 855 |
| *T. thiocyanoxidans* NCIB 8572 | 20 | 7.37 | 7.01 | 232 | 0 | 196 | 50 | 58 | 647 |

1) A small amount of gas formed, but composition not determined.

aerobically and anaerobically in the presence of nitrate. The unsuccessful attempts to grow *T. thiooxidans* in a medium containing thiocyanate at pH 4.8 suggest, however, that not all *Thiobacillus* species can utilise this substance. Youatt has suggested that the first step in the utilisation of thiocyanate is its enzymatic breakdown to cyanate and hydrogen sulphide. Since Parker and Prisk (1953) have shown that *T. thiooxidans* can readily utilise hydrogen sulphide as sole sulphur source, its inability to utilise thiocyanate may be due to lack of the thiocyanate hydrolysing enzyme system.

The second important result of the present work is the demonstration that three strains of *T. thioparus*, including the type strain, and Happold's strain of *T. thiocyanoxidans* can grow anaerobically in the presence of nitrate with either thiosulphate or thiocyanate as sulphur source. Up to now, *T. thioparus* has generally been regarded as capable of growing only under aerobic conditions (Bergey, 1948), although Beijerinck reported the presence of this species in anaerobic enrichment cultures for *T. denitrificans*. He stated, however, that it was incapable of denitrification. It is not clear what means he employed to determine whether denitrification had occurred, but if his criterion was production of gas, his observations do not conflict with ours, since we found no gas production with *T. thioparus* either in thiosulphate or thiocyanate medium.

*T. thioparus* and *T. thiocyanoxidans* differ from *T. denitrificans* in the stage to which they are able to reduce nitrate. With thiocyanate as reductant, as with thiosulphate, *T. denitrificans* carries the reduction as far as $N_2$. Under our conditions, little or none of the intermediate products, which have been shown by BAALSRUD and BAALSRUD to include nitrite, nitric oxide and nitrous oxide, accumulated in the medium. With *T. thioparus* and *T. thiocyanoxidans* the reduction in the presence of thiocyanate stopped when the nitrate had been reduced to nitrite. No gaseous reduction products were produced. In thiosulphate medium the picture was somewhat more complex. The principal reduction product found was again nitrite. *T. thiocyanoxidans*, however, also produced a small amount of nitrogen. With *T. thioparus*, only part of the nitrate reduced was found as nitrite. The rest could not be accounted for. In view of the analytical difficulties, it is uncertain how significant this deficit is. Further work on the reduction of nitrate by this species is desirable.

The results suggest that *T. thioparus* and *T. thiocyanoxidans* are deficient in or lacking the enzyme system which is responsible for the reduction of nitrite by *T. denitrificans*. This suggestion is supported by the results of BAALSRUD and BAALSRUD who showed that cell suspensions of *T. thioparus* were unable to reduce nitrite in the presence of thiosulphate and air whereas suspensions of *T. denitrificans* reduced it rapidly, first to nitric oxide and finally to nitrogen.

In view of our findings it is necessary to reconsider the relationships between *T. denitrificans*, *T. thioparus* and *T. thiocyanoxidans*.

HAPPOLD and KEY, and HAPPOLD, JOHNSTONE, ROGERS and YOUATT based the creation of the species *T. thiocyanoxidans* on the ability of their strains to oxidise thiocyanate which they regarded as unique. Our observations, as well as those of BEIJERINCK, show that ability to utilise thiocyanate is not in itself sufficient to justify the creation of a separate species. We found no difference between *T. thiocyanoxidans* and *T. thioparus* with regard to ability to utilise thiocyanate either aerobically or anaerobically in the presence of nitrate. The only difference found between the two species was the minor difference in the products of nitrate reduction in the presence of thiosulphate. As has been pointed out, it is uncertain to what extent these differences are real. Initially some cultural differences were observed between the two species. When *T. thiocyanoxidans* was first cultured in liquid medium containing thiosulphate, it failed to develop the sulphur pellicle which is typical of *T. thioparus*. By repeated transfers from the surface of such liquid cultures, however, this rather characteristic pellicle formation could be induced to such an extent that any macromorphological differences between cultures of the two species became non-existent.

In view of these facts, it is considered that *T. thiocyanoxidans* cannot be regarded as a separate species, but that it is identical with, or at the most a variant of *T. thioparus*.

The ability to oxidise sulphur and thiosulphate to sulphate under anaerobic conditions with nitrate as hydrogen accepter has been regarded as a distinctive characteristic of *T. denitrificans* (BERGEY). This ability has now been shown to be possessed also by *T. thioparus* (which species is taken to include *T. thiocyanoxidans*).

There is a clear difference, however, in the stage to which *T. denitrificans* and *T. thioparus* can carry the reduction of nitrate. There is also a difference in the appearance of the colonies when grown aerobically on thiosulphate agar.

The maintenance of *T. denitrificans* and *T. thioparus* as separate species may thus be justified, although *T. thioparus* could also be regarded as a metabolic variant of *T. denitrificans* in which the ability to reduce nitrite has been largely lost.

## Summary.

*Thiobacillus denitrificans*, *T. thioparus* and *T. thiocyanoxidans* can oxidise thiocyanate to sulphur and sulphate aerobically and also anaerobically in the presence of nitrate.

*T. thioparus* and *T. thiocyanoxidans* are capable of growing anaerobically in media containing nitrate and either thiosulphate or thiocyanate. The reduction of nitrate by these species stops largely at the stage of nitrite and little or no gaseous reduction products are formed. This contrasts with the behaviour of *T. denitrificans* which reduces nitrate ultimately to nitrogen.

On the basis of these results, it is suggested that *T. thiocyanoxidans*

is identical with *T. thioparus*. The maintenance of *T. denitrificans* and *T. thioparus* as separate species can be justified, although the latter could be regarded as a metabolic variant of the former.

## Acknowledgement.

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# MOLLISIN

## A PRELIMINARY EXAMINATION OF ITS ANTIBIOTIC ACTIVITY

by

**M. M. NIJLAND** and **O. F. UFFELIE**

(Received February 2, 1957).

Some time ago we received from J. GREMMEN (Forest Research Station T.N.O., Wageningen) two cultures of species of *Mollisia*, designated 69a and 71b; these moulds were later identified by GREMMEN (1956) as *Mollisia caesia* Sacc. sensu Sydow and *M. fallens* (Karst.) Karst. This author had observed that on malt agar yellow crystals were formed around the colonies and that these crystals formed a barrier impeding further growth of the mould. This observation had our interest because chemical products of Discomycetes have only been sporadically investigated and those of the *Mollisioideae* not at all.

Large scale cultures on malt agar were made of both strains and in either case the yellow substance was produced. This substance was isolated by pouring chloroform onto the plates on which the mould had grown for 2—3 weeks. After evaporation of the chloroform the residue was dissolved in acetone and the yellow substance precipitated by addition of water. The substance which was still amorphous at this stage, was again dissolved in acetone and, after spontaneous evaporation of the latter, reappeared as a beautiful felty crystalline, orange yellow mass, m.p. 194° C.

As both strains appeared to produce the same substance, further experiments were carried out with strain 71b, which was a better producer than strain 69a.

The yellow substance is almost insoluble in water, but dissolves in alkali yielding a red brown solution. Nitrogen appeared to be absent, but a considerable amount of chlorine was found. We called the compound that had not been described before mollisin.

## TABLE 1.

Antibiotic activity of mollisin.

| | 0 µg | 0.003 µg | 0.01 µg | 0.03 µg | 0.1 µg | 0.3 µg | 1 µg | 3 µg | 10 µg | 30 µg | 100 µg | 300 µg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Micrococcus pyogenes* var. *aureus* coag. + | — | — | — | + | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *Micrococcus pyogenes* var. *aureus* coag. — | — | — | — | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| *Micrococcus pyogenes* var. *aureus* coag. — [1] | — | — | — | — | ± | + | + | + | + | + | + | |
| *Micrococcus lysodeikticus* | — | — | — | — | ++ | ++ | +++ | +++ | +++ | +++ | +++ | ++++ |
| *Sarcina flava* | — | — | — | + | ++ | ++ | +++ | +++ | ++ | ++ | +++ | +++ |
| *Neisseria catarrhalis* (Levinthal-agar) | — | — | — | — | — | + | + | + | + | ++ | | |
| *Streptococcus faecalis* | — | — | — | — | — | + | + | + | ++ | ++ | ++ | ++ |
| ,, ,, [1] | — | — | — | — | — | + | + | + | + | + | + | + |
| *Lactobacillus casei* (Rogosa-plate) | — | — | — | — | — | + | ++ | ++ | ++ | ++ | ++ | |
| *Alcaligenes faecalis* | — | — | — | — | — | — | — | — | — | — | — | — |
| *Escherichia coli* | — | — | — | — | — | — | — | — | — | — | — | — |
| *Klebsiella* spec. | — | | | — | — | — | — | — | — | — | | |
| *Serratia marcescens* | — | — | — | — | — | — | — | — | — | — | — | — |
| *Proteus vulgaris* | — | | | — | — | — | — | — | — | — | | |
| *Bacillus subtilis* | — | — | — | + | ++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| *Bacillus megatherium* | — | — | — | — | ++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| ,, ,, [1] | — | — | — | — | ± | + | + | + | + | + | + | + |
| *Bacillus cereus* | — | — | — | + | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| ,, ,, [1] | — | — | — | — | — | — | — | — | — | — | | |
| *Bacillus mycoides* | — | — | + | + | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| *Mycobacterium phlei* | — | — | — | — | — | + | ++ | +++ | +++ | +++ | +++ | +++ |

[1] on blood agar.

Legend: Peptone yeast extract broth plates were used unless otherwise indicated. Diameter of disc 5.7 mm.

| | | |
|---|---|---|
| Diameter of disc + inhibition zone | 5.7 mm | — |
| ,, ,, | 5.7 — 6.0 ,, | ± |
| ,, ,, | 6.1 — 8.0 ,, | + |
| ,, ,, | 8.1 — 10.0 ,, | ++ |
| ,, ,, | 10.1 — 12.0 ,, | +++ |
| ,, ,, | > 12.0 ,, | ++++ |

For further chemical investigation and large scale cultivation of the moulds both strains were handed to the Institute for Organic Chemistry T.N.O., Utrecht (VAN DER KERK and OVEREEM, 1957).

Mollisin was expected to show antibiotic activity, because the crystals slowed down the growth of *Mollisia* itself and of other moulds. It appeared also to display antibiotic activity towards *Micrococcus pyogenes* var. *aureus*.

### Antibiotic activity of mollisin.

As mollisin is only slightly soluble in water, its antibiotic activity could not be investigated by the cylinder-plate method, the cup-plate method or the dilution method. This would be feasible, if mollisin were dissolved beforehand in propylene glycol (in which it is slightly soluble), but we preferred the paper-disc method which renders additions superfluous. Whatman Nr. 1 filter paper discs of 5.7 mm diameter were used. By means of a micropipette acetonic solutions of mollisin were carefully applied to the pinned up paper discs giving amounts of 0.003—300 µg mollisin per disc; the acetone was allowed to evaporate. The discs were placed onto peptone yeast extract broth plates, which had been inoculated with bacteria cultivated on liquid media of the same composition. The plates were incubated at 37°C. during 18 hrs.

At request of Dr G. J. M. VAN DER KERK, Institute for Organic Chemistry T.N.O., the activity of mollisin was compared with that of the chemically related compound 2,3-dichloronaphthaquinone which is a well-known fungicide ("phygon"). The results are summarized in Tables 1 and 2.

TABLE 2.
Antibiotic activity of 2,3-dichloronapthaquinone.

| | 0 µg | 0.003 µg | 0.01 µg | 0.03 µg | 0.1 µg | 0.3 µg | 1 µg | 3 µg | 10 µg |
|---|---|---|---|---|---|---|---|---|---|
| *Micrococcus pyogenes* var. *aureus* coag. + | — | — | — | — | — | + | ++ | ++ | ++ |
| *Escherichia coli* | — | — | — | — | — | — | — | — | — |
| *Bacillus megatherium* | — | — | — | — | — | + | + | ++ | ++ |

Legend: see Tabel 1.

## Summary.

From cultures of *Mollisia fallens* and *M. caesia* a yellow, crystalline substance was isolated which contains chlorine but is free of nitrogen; it has been named mollisin.

Mollisin shows strong antibiotic activity towards Gram-positive bacteria and *Neisseria catarrhalis* and proved inactive towards the Gram-negative rods investigated. The activity is strongly counteracted by blood. 2,3-dichloronaphthaquinone shows comparable but weaker activity.

We are deeply indebted to Professor K. C. Winkler and Mr. A. Potasse for advice and for provision of laboratory facilities for the bacteriological part of this investigation.

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# GENETIC RECOMBINATION IN *E. COLI* B

## PART IV [1]. THE SEQUENCE OF SOME UNSELECTED MARKERS

by

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(Received July 19, 1957).

### INTRODUCTION.

In a previous paper (2, 3) recombination experiments with *E. coli* B have been described. In these experiments the loci/15 and /34, governing the cross resistance to the phages $T_1$ and $T_5$ or $T_3$ and $T_4$ respectively, were used as unselected markers. A comparision of the class of methionine-auxotrophic recombinants with the prototrophic progeny seemed to indicate that the loci for phage resistance were both linked to the methionine locus. The low interaction between the unselected loci seemed to refute this idea. Several other discrepancies were noted.

This paper presents additional data and shows conclusively that the loci /15 and /34 are linked to the methionine locus. Furthermore the order of sequence of a group of five unselected markers was established.

The bearing of the experimental results on possible mechanisms of recombination in *E. coli* B will be discussed.

### MATERIALS AND METHODS.

The parental strain in all cases is *Escherichia coli* B. Auxotrophic mutants were obtained by ultraviolet irradiation. The following auxotrophic markers were used: methionine (m), tyrosine (ty), histidine (h), tryptophane (tr), pantothenic acid (pa) and leucine (l). Two unselected markers, governing the cross resistance against the bacteriophages $T_1$ and $T_5$ (/15c) or $T_3$ and $T_4$ (/34a) were used.

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[1] Parts I, II, III: Genetica **27**, 293, 300, 1954; **27**, 364, 1955.

Resistant and sensitive recombinants are denoted by $R_1$ and $R_3$ or $S_1$ and $S_3$ respectively.

The /15c marker was also used as a selective marker by spraying the recombination plates with phage $T_1$ after 5 hours incubation at 37° C. The bacterial strains used are:

a) strain 609F$^+$h$^-$ tr$^-$/34a/15c
b) strain 615F$^-$m$^-$ty$^-$
c) strain 615–11–2 F$^-$m$^-$ty$^-$pa$^-$l$^-$

Media and recombination techniques were the same as described before (3). Unselected markers were scored with the replica technique of LEDERBERG and LEDERBERG (6).

## RESULTS.

In previous experiments the cross 609F$^+$/34a/15c × 615F$^-$ had produced a prototrophic progeny with 22% $T_1$ and 51% $T_3$ resistant colonies (3). The loci /34a and /15c showed some linkage with the m locus, as the transfer of the F$^+$ alleles to the m$^-$ recombinant class was much smaller. The interaction between /34a and /15c was low (1.38) and not significantly greater than unity, suggesting that both markers were only weakly linked. No sequence of the three loci (/15c, /34a and m) could be determined without additional data.

Two other markers (pa$^-$ and l$^-$) were therefore introduced in strain 615F$^-$ (615–11–2 F$^-$).

The linkage between the markers /15c, pa, l, m and /34a was studied by crossing strain 609F$^+$/34a/15c with strain 615–11–2 F$^-$. A number of recombinant classes were selected by supplementing the plates with various combinations of growth factors or spraying with phage $T_1$ (table 1).

TABEL 1.

Cross 609 F$^+$h$^-$tr$^-$/34a/15c × 615–11–2 F$^-$m$^-$ty$^-$pa$^-$l$^-$ on media supplied with all growth factors except those corresponding with the selected markers (see column 1).

| Selected recombinant class | Number of recombinants | Fraction of F$^+$ allele | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | h$^-$ | tr$^-$ | $R_1$ | pa$^+$ | l$^+$ | m$^+$ | $R_3$ |
| h$^+$ty$^+$ | 840 | | 0.073 | 0.033 | 0.037 | 0.037 | 0.040 | 0.030 |
| tr$^+$ty$^+$ | 1039 | 0.161 | | 0.081 | 0.100 | 0.097 | 0.090 | 0.046 |
| h$^+$tr$^+$ty$^+$ | 1900 | | | 0.067 | 0.066 | 0.075 | 0.068 | 0.037 |
| h$^+$tr$^+$ty$^+$/$R_1$ | 220 | | | | 0.859 | 0.782 | 0.372 | 0.286 |
| h$^+$tr$^+$ty$^+$pa$^+$ | 892 | | | 0.873 | | 0.796 | 0.328 | 0.236 |
| h$^+$tr$^+$ty$^+$l$^+$ | 840 | | | 0.774 | 0.809 | | 0.350 | 0.245 |
| h$^+$tr$^+$ty$^+$m$^+$ | 644 | | | 0.209 | 0.224 | 0.231 | | 0.588 |

It was found that a low fraction of the F$^+$ alleles is transferred to the h$^+$ty$^+$, tr$^+$ty$^+$ or h$^+$tr$^+$ty$^+$ progeny. A much larger fraction of the F$^+$ alleles however is found if additional selection is made for one of the loci /15c, pa$^+$, l$^+$ or m$^+$, indicating that the loci /15c, pa, l, m and /34a belong to one linkage group.

The argument is not quite valid however as the data from table 1 are obtained under different experimental conditions. It was shown previously, that a change in experimental conditions, e.g. the addition of methionine to the plates can alter the transfer. In this case it is obviously not so, as can be seen in table 2, where the various recombinant classes obtained in one experiment (the experiment from the 3rd row in table 1) are given separately.

If f.i. the locus /15c is not transferred, only 1% of the sensitive recombinants are non auxotrophic for pantothenic acid, (pa$^+$) against 86.5% when the recombinants are T$_1$ resistant etc. Obviously the five markers belong to one linkage group.

TABLE 2.

Cross 609 F$^+$h$^-$tr$^-$/34a/15c × 615–11–2 F$^-$m$^-$ty$^-$pa$^-$l$^-$ on minimal medium, supplemented with pantothenic acid, leucine and methionine. Selection was made for h$^+$, tr$^+$ and ty$^+$. The 1900 recombinants were afterwards divided (five times) in two groups.

| Recombinant class | Number of recombinants | Fraction of F$^+$ allele | | | | |
|---|---|---|---|---|---|---|
| | | R$_1$ | pa$^+$ | l$^+$ | m$^+$ | R$_3$ |
| S$_1$ | 1773 | | 0.010 | 0.025 | 0.047 | 0.023 |
| R$_1$ | 127 | | 0.865 | 0.772 | 0.370 | 0.229 |
| pa$^-$ | 1774 | 0.010 | | 0.024 | 0.048 | 0.023 |
| pa$^+$ | 126 | 0.865 | | 0.801 | 0.349 | 0.231 |
| l$^-$ | 1757 | 0.017 | 0.014 | | 0.038 | 0.021 |
| l$^+$ | 143 | 0.685 | 0.706 | | 0.447 | 0.237 |
| m$^-$ | 1770 | 0.045 | 0.047 | 0.044 | | 0.011 |
| m$^+$ | 130 | 0.361 | 0.338 | 0.492 | | 0.392 |
| S$_3$ | 1830 | 0.053 | 0.053 | 0.059 | 0.010 | |
| R$_3$ | 70 | 0.414 | 0.414 | 0.483 | 0.729 | |

The sequence of the five markers can be determined from table 1 or table 2. Selection for R$_1$ gives us two possible sequences for the markers /15c, pa and l, namely 15c–pa–l or pa–15c–l. Selection for l$^+$ on the other hand indicates that the only possible sequence can be 15c–pa–l. Continuing along this line, the sequence of the five markers is: 15c – pa – l – m – 34a (Fig 1).

It is again clear that one must seriously distrust the absolute value of transfer-percentages under different experimental conditions. If selection is made for $m^+$ by omitting methionine from the recombination plates (table 1 last row) 58.8% of the $m^+$ recombinants are resistant against phage $T_3$; when the cross is done on plates supplied with methionine and the $m^+$ recombinants are scored afterwards (table 2 last column) only 39.2% is resistant against $T_3$.

Although nothing can be said about the (relative) distances between the five loci, it is evident that the distance between 15c and pa is the shortest one, the distance l – m being somewhat greater etc. (Fig. 1)

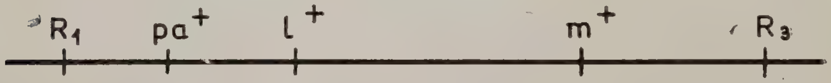

Fig. 1. Diagram of position and relative distances in the linkage group.

### The interaction between unselected markers.

The interaction 1) between two unselected markers could be a measure for the degree of linkage between the markers studied. In this linkage group however we must distinguish between the interaction, obtained when the ($F^+$) selective marker is located between the unselected markers and an interaction between two unselected markers located on the same side of the ($F^+$) selective marker.

### Interaction between two unselected markers on the same side of the selection point.

Whatever mechanism of transfer would be operative, one would in first instance expect the interaction to depend only on the distance of the unselected markers, the shorter the distance, the higher the interaction.

The four first rows of table 3 show that indeed the interaction decreases with increasing distance between the unselected markers. However the site of the selection point also seems to play a role. The

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1) Interaction equals the product of the parental frequencies, divided by the product of the crossover frequencies. If the interaction is significantly greater than unity ($X^2$ test), the markers are linked.

## TABLE 3.

Cross 609 $F^+h^-tr^-/34a/15c \times 615\text{–}11\text{–}2\ F^-m^-ty^-pa^-l^-$.

Interaction (I) is given between the locus at the head of each section and the loci at the head of the columns in each section. This arrangement means that some data appear twice in the table. The upper three rows refer to selection points outside the group, the last four rows to selection points within the group. Interactions between loci on different sides of selection points are in heavy print.

The probability of independent transfer is given in the form of $X^2$. Values of $X^2 = 4.6, 7.8, 9.2$ correspond to probabilities of independent transfer of $P = 0.1, 0.02$ and $0.01$ respectively

| Selected combinant(s) class | Number of re-combinants | | Interaction and probability of independent transfer between | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | h | | | | | tr | | | | | $R_1$ | | | | pa | | | | l | | | | m | | | |
| | | | $R_1$ | pa | l | m | $R_3$ | $R_1$ | pa | l | m | $R_3$ | pa | l | m | $R_3$ | $R_1$ | l | m | $R_3$ | $R_1$ | pa | m | $R_3$ | $R_1$ | pa | l | $R_3$ |
| h+ty+ | 840 | I | | | | | | 2.9 | 3.3 | 3.3 | 2.8 | 3.4 | 240 | 240 | 31 | 29 | 240 | 168 | 18 | 16 | 240 | 168 | 53 | 59 | 31 | 18 | 53 | 452 |
| | | $X^2$ | | | | | | 5.0 | 6.7 | 6.7 | 5.1 | 6.3 | 429 | 429 | 126 | 113 | 429 | 354 | 78 | 60 | 429 | 354 | 206 | 204 | 126 | 78 | 206 | 473 |
| tr+ty+ | 1039 | I | 1.8 | 1.6 | 1.6 | 1.1 | 1.1 | | | | | | 1739 | 214 | 20 | 59 | 1739 | 190 | 18 | 53 | 214 | 190 | 28 | 45 | 20 | 18 | 28 | 88 |
| | | $X^2$ | 6.9 | 5.4 | 4.3 | 0.4 | 0.3 | | | | | | 780 | 624 | 212 | 302 | 780 | 635 | 207 | 267 | 624 | 635 | 286 | 255 | 212 | 207 | 286 | 341 |
| h+tr+ty+ | 1900 | I | | | | | | | | | | | 626 | 130 | 12 | 13 | 626 | 167 | 11 | 12 | 130 | 167 | 21 | 15 | 12 | 11 | 21 | 59 |
| | | $X^2$ | | | | | | | | | | | 1382 | 955 | 197 | 140 | 1382 | 1021 | 167 | 140 | 955 | 1021 | 352 | 175 | 197 | 167 | 352 | 496 |
| h+tr+ty+$R_1$ | 220 | I | | | | | | | | | | | | | | | | 79 | 2.3 | 4.3 | | 79 | 2.1 | 3.5 | | 2.3 | 2.1 | 38 |
| | | $X^2$ | | | | | | | | | | | | | | | | 86 | 3.4 | 6.4 | | 86 | 4.0 | 7.7 | | 3.4 | 4.0 | 89 |
| h+tr+ty+pa+ | 892 | I | | | | | | | | | | | | **7.2** | **1.7** | **2.7** | | | | | 7.2 | | **4.2** | **2.5** | **1.7** | | **4.2** | 10 |
| | | $X^2$ | | | | | | | | | | | | 105 | 5.1 | 11 | | | | | 105 | | 41 | 16 | 5.1 | | 41 | 198 |
| h+tr+ty+l+ | 840 | I | | | | | | | | | | | 106 | | **1.6** | **2.0** | 106 | | **2.0** | **1.5** | | | | | **1.6** | **2.0** | | 14 |
| | | $X^2$ | | | | | | | | | | | 524 | | 7.6 | 11 | 524 | | 13 | 3.4 | | | | | 7.6 | 13 | | 243 |
| h+tr+ty+m+ | 644 | I | | | | | | | | | | | 116 | 57 | | **1.5** | 116 | 63 | | **1.6** | 57 | 63 | | **1.8** | | | | |
| | | $X^2$ | | | | | | | | | | | 407 | 336 | | 3.9 | 407 | 351 | | 5.4 | 336 | 351 | | 8.7 | | | | |

data of the last column of table 3 (interaction between m and R) leave no doubt that the interaction decreases from top to bottom i.e. that the interaction decreases the more the selection point nears the unselected markers.

The highest interaction (452) is found among the $h^+ty^+$ recombinants (selection point far away); the smallest interaction (10,14) among the $h^+tr^+ty^+pa^+$ and $h^+tr^+ty^+l^+$ recombinants, i.e. with the selection point very near.

The interaction seems to vary inversely with the distance between the unselected markers but also seems to vary directly with the distance from the selection point.

All interactions between /34a and the other loci are somewhat higher than expected from the trend of the data for other loci. This might be due to the influence of an assumed $F^-$ selection point ($h^+$)?

**Selective marker between unselected markers.**

Whatever mechanism of transfer would be operative, complete chromosomes and normal crossover, random breaking of the $F^+$ chromosomes and transfer of pieces to the gene acceptor strain etc., one would expect that the interaction between two markers would not significantly differ from unity, when the selective marker (on the $F^+$ or on the $F^-$ chromosome) is located between the two unselected markers.

This expectation is not fulfilled, as can be seen in table 3. When selection is made for $pa^+$ the interaction between /15 and 1 (7.2) differs significantly from unity ($P \leqq 0.001$). In this case the distance between the markers is small, giving a high interaction. If the distance becomes greater (15 c–m) the interaction decreases. If the distance becomes still greater (15a – /34a) the interaction has a tendency to increase. It is however always greater than unity and the probability of independent transfer is always small ($P < 0.1$).

The explanation for the unexpected high interaction between /15c and 1 could be caused by too high a fraction of parental types or (and) by too low a fraction of crossover types. In table 4 the fraction of the four recombinant classes for /15c – 1 and /15c – m (selective marker $pa^+$), obtained in one experiment are given.

Considering that the distance between pa and m is far greater than the distance pa – 1, the data of table 4 show that the fraction $S_1$-$pa^+l^-$ (0.073) is far in excess, when compared with the fraction $S_1$-$pa^+m^-$ (0.097). This discrepancy is not due to a high fraction of

TABLE 4.

Cross 609 F$^+$h$^-$tr$^-$/34a/15c × 615–11–2 F$^-$m$^-$ty$^-$pa$^-$l$^-$.

Selection was made for h+, tr+, ty+ and pa+ on medium supplied with leucine and methionine. The total population of 892 recombinants was scored for unselected markers and grouped in four classes in respect to the pairs 15c–l or 15c–m.

| Recombinant class | | Number of recombinants | fraction |
|---|---|---|---|
| 15c | l | | |
| $R_1$ | l$^+$ | 659 | 0.739 |
| $R_1$ | l$^-$ | 120 | 0.134 |
| $S_1$ | l$^+$ | 49 | 0.054 |
| $S_1$ | l$^-$ | 64 | 0.073 |
| 15c | m | | |
| $R_1$ | m$^+$ | 269 | 0.301 |
| $R_1$ | m$^-$ | 510 | 0.572 |
| $S_1$ | m$^+$ | 27 | 0.030 |
| $S_1$ | m$^-$ | 86 | 0.097 |

double crossovers as the fraction $S_1$–pa$^+$–l$^-$m$^+$ is very small (0.009).

It seems as if a high fraction of the recombinant class $S_1$ – pa$^+$, having a crossover between $S_1$ and pa$^+$ also have a crossover between the loci pa$^+$ and l$^-$, giving an unusual high fraction of the recombinant class $S_1$–pa$^+$–l$^-$, resulting in a high interaction between the loci $R_1$ and pa. A similar phenomenon has been described by ROTHFELLS (7) and called negative interference. The phenomenon might also be explained by assuming, that only small pieces of the F$^-$ chromosome are either given by the donor or incorporated by the acceptor strain.

**Selection of complementary recombinants.**

During the course of the experiments it was thought that selection of complementary recombinants could teach something about the transfer-mechanism. Complementary recombinants were selected in the following way: strain 609 F$^+$h$^-$tr$^-$/34a/15c and 615–11–2 F$^-$m$^-$ty$^-$pa$^-$l$^-$ were crossed on minimal medium, supplemented with either h and pa or tr and ty. Afterwards the recombinants were scored for the markers tr and ty or h and pa respectively. The recombinant classes tr$^+$ty$^+$h$^-$pa$^-$ and tr$^-$ty$^-$h$^+$pa$^+$ were further scored for the unselected markers /15c, l, m and /34a. Data and figures of a typical experiment are given in figure 2.

Figure 2 shows that the selected (complementary) recombinants are not complementary for the unselected markers. In accordance

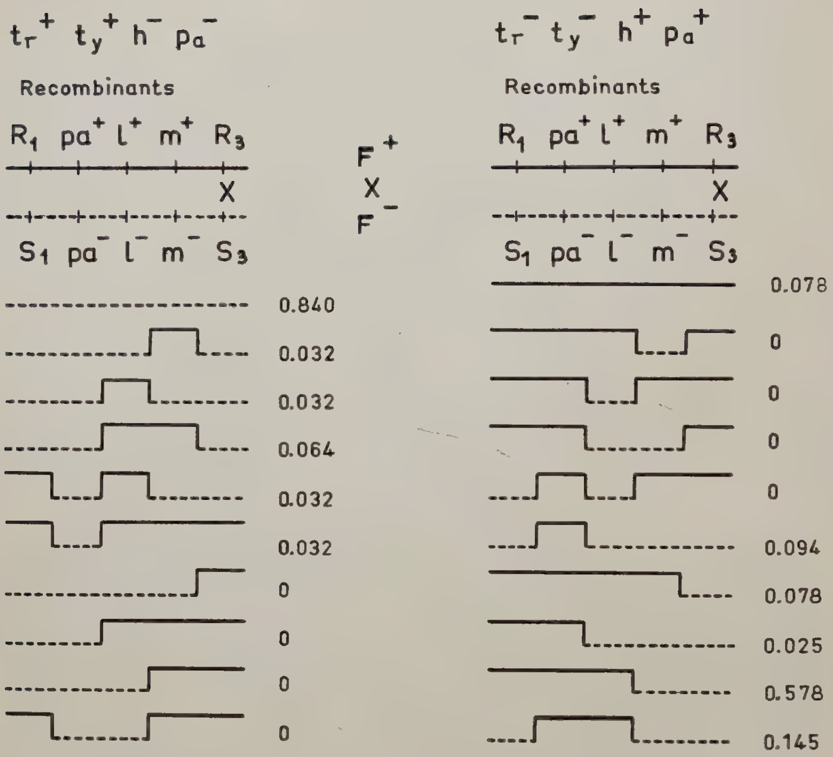

Fig. 2. Cross 609 $F^+h^-tr^-/15c/34a$ × 615–11–2 $F^-m^-ty^-pa^-l^-$. The fraction of the various recombinants in the complementary classes $tr^+ty^-h^-pa^+$ and $tr^-ty^+h^+pa^-$.

with earlier experiments a high fraction of the $tr^-ly^-h^+pa^+$ recombinants is of the $R_1l^+m^-S_3$ type, the complementary recombinant $tr^+ty^+h^-pa^-S_1l^-m^+R_3$ is not found at all, etc. These data seem to indicate that small parts of the $F^+$ chromosome are incorporated in the chromosome of the recombinants. This is also seen in fig. 3, in which the separate data of 892 recombinants of the $h^+tr^+ty^+pa^+$ class are given.

## Summary and discussion.

In previous papers (2,3) it was shown that in *E. coli* B the resistance to the phages $T_1$–$T_5$ and $T_3$–$T_4$ respectively, were linked to the methionine marker. The interaction between the loci /15c and /34a was low, however. As this discrepancy, low interaction and linkage to the same selected marker could be due to the place of the

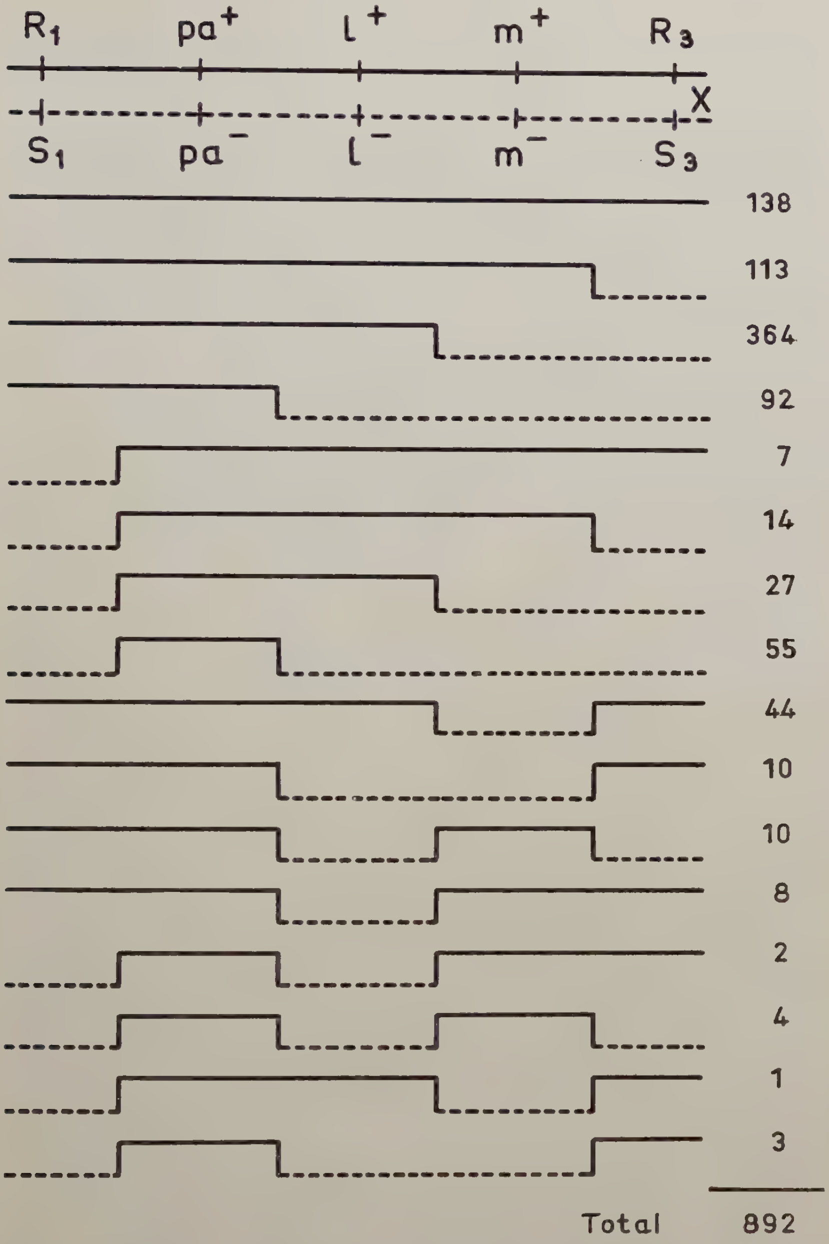

Fig. 3. Cross 609 $F^+h^-tr^-/15c/34a \times 615\text{–}11\text{–}2\ F^-m^-ty^-pa^-l^-$. Selection for $h^+tr^+ty^+pa^+$. The frequency of the various combinations of unselected markers are given in the table.

selected marker, additional data with nearby markers were necessary.

Two additional markers (pa$^-$ and l$^-$) were therefore introduced in strain 615 F$^-$ (m$^-$ty$^-$) giving strain 615–11–2 F$^-$(m$^-$ty$^-$pa$^-$l$^-$). It was found that the loci /15c, pa, l, m and /34a belong to one linkage group and the sequence in the linkage group was determined (table 1 and 2 and fig. 1). In fact all loci are linked to each other, and apparently are on one chromosome. The interaction between unselected markers was calculated (table 3). It was found, that the absolute value of the interaction depends on two factors, the place of the unselected markers in respect to the selection point and the distance between the unselected markers.

If the selection point is located between two unselected markers no interaction was expected. Though the observed interactions are not large they were always present and increased with decreasing distance between the unselected markers. If the unselected markers are on the same side of the selection point the interaction decreases when the distance between the unselected markers increases. Peculiarly however the interaction decreased with decreasing distance from the selected markers. Thus linkage between unselected markers decreases when the selection point comes nearer.

It seems difficult to imagine a mechanism of recombination to explain these results. It was thought that the isolation of complementary recombinants might help to elucidate the transfer mechanism, operative in *E. coli* B, as data about diploid segregation etc. are not yet known for this organism.

It was found (fig. 2) that although the recombinants are complementary for 4 markers, they are not complementary at all for the unselected markers. This kind of experiment excludes a transfer mechanism whereby the complete chromosome set of the gene donor forms a normal diploid with the acceptor, with segregation after normal crossover. The relatively large number of cases in which only one or two separate loci from a group are transferred rather seems to point to the transfer of small pieces of the donor chromosome.

Analogous abnormalities have been found with *E. coli* K$_{12}$. Jacob and Wollman (5) showed for this organism that the transfer of genetic material from donor (F$^+$) to recipient cell is the result of a mutation to high frequency of recombination. With the isolated high frequency (Hfr) strains they found that the transfer of genetic material was time-dependent, some loci being transferred earlier than others. They explained this by supposing, that the transfer mechanism started from a certain point on the $F^+$ chromosome and proceeded along the chromosome, reaching each locus at a given time. This high frequency transfer was however always restricted to a small part of the chromosome. The same results were obtained by HAYES (4) who furthermore showed that in normal $F^+ \times F^-$ crosses on analogous time dependency occurs. The transfer of genetic material from the donor cell requires energy (FISHER (1)). The recipient cell however does not require a high energy reserve to accept and integrate the genetic material. JACOB and WOLLMAN showed for Hfr × $F^-$ crosses that only a small part of the transferred genetic material is integrated in the chromosome of the recipient cell and contributes to the recombinant.

It is of course possible that many of the phenomena observed with $K_{12}$ can also occur in *E. coli* B. Mutation to Hfr and time dependency as described for *E. coli* $K_{12}$ do not seem to explain our results with *E. coli* B completely however.

F.i. in the cross 609 $F^+h^-tr^-$/15/34a × 615 $F^-m^-ty^-pa^-l^-$ the recombinants were always selected for a marker ($ty^+$) far outside the linkage group $R_1$–pa–l–m–$R_3$. In many cases apart from the $ty^+$ locus only one or two loci of this linkage group were transferred (fig. 2 and 3). This seems to show that at least two small parts of the $F^+$ chromosome contributed to the recombinants.

There obviously exist two possibilities. Several small parts of the $F^+$ chromosome are transferred in *E. coli* B, and the mechanism of transfer would be different from that in *E. coli* $K_{12}$. On the other hand the mechanism of transfer might be the same in both organisms but during integration the transferred segment would be broken up into smaller pieces in *E. coli* B than in *E. coli* $K_{12}$. This seems to leave us with the old problem in bacterial genetics: how to explain the results without accepting an unduly large number of crossovers or breaks.

Different Hfr mutations and time dependency do not overcome this difficulty.

## Acknowledgement.

The author is greatly indebted to Prof. Dr K. C. WINKLER for his constant interest and valuable suggestions and to Miss H. S. FELIX for valuable technical assistance.

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# THE CATABOLISM OF LACTOSE AND LACTOBIONATE BY *CORYNEBACTERIUM SIMPLEX*

by

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(Received July 23, 1957).

## INTRODUCTION.

STODOLA and LOCKWOOD (1947) discovered that several species of the genus *Pseudomonas* are able to oxidize maltose and lactose to the corresponding bionic acids. KLUYVER, DE LEY and RIJVEN (1950) confirmed and extended these observations. As the first step in the decomposition of a disaccharide is usually a hydrolysis, a phosphorolysis or the linkage of a hexose moiety to a transferase, the formation of bionic acids by these highly aerobic organisms represents the only known aberrant mechanism in disaccharide metabolism, whereby the oxygen bridge remains intact.

Very little is known on the biochemical and enzymatic mechanism of the decomposition of these compounds. It has been shown that $\beta$-galactosidase or $\alpha$-glucosidase is able to hydrolyze slowly the oxygen bridge of the corresponding bionic acids (for a review of the literature, see DE LEY and BERNAERTS, 1952). However, the physiological meaning of this hydrolysis has not yet been evaluated, as no pathways for bionate catabolism have been studied. BERNAERTS (1953) and BERNAERTS and DE LEY (1952, 1953) have reported that a great number of micro-organisms cause a rapid and very efficient decomposition of both bionates. During an extensive biochemical and enzymatic study of its mechanism with several strains of this collection, it became obvious that at least two entirely

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different pathways exist. One of these pathways consisted in the hydrolysis of lactose and lactobionate by the same $\beta$-galactosidase. It was encountered in a strain of *Corynebacterium simplex*, and is the subject of the present paper.

## EXPERIMENTAL.

1. Bacteria used. A very active strain was isolated from lactobionate plates, inoculated with river water. It was identified as a member of the *Corynebacteriaceae* and exhibits the vast majority of characteristics reported in Bergey's Manual, 6th Ed., for *C. simplex*. However, the nitrate reduction is weak and difficult to establish; neither is the growth abundant at 37°C. This strain has been maintained in the laboratory for several years on a medium, containing lactobionate as main carbon source.

2. Growth media for the study of lactose and lactobionate decomposition contained the following ingredients : 3% potassium lactobionate or lactose, 0.2% $(NH_4)_2SO_4$, 0.1% $KH_2PO_4$, 0.025% $MgSO_4 . 7 H_2O$, 0.175% yeast extract (Difco), 1% $CaCO_3$; final pH 6.8. The carbohydrates were sterilized by filtration. The medium was distributed in Erlenmeyer flasks in shallow layers, inoculated with a suspension of young cells and the cultures incubated at 30°C. on a shaking device. Every day a sample was taken out with a sterile pipet, centrifuged and the pH and concentration of carbohydrates determined in the supernatant.

3. Calcium lactobionate was prepared by means of electrolytic oxidation according to ISBELL and FRUSH (1931, 1933, 1936). The potassium salt was obtained, after removal of Ca by oxalic acid, by neutralisation with KOH.

4. Preparation of autolyzed cells. The bacteria were grown for 20 hours in the previously described medium without $CaCO_3$ and 1% lactose or lactobionate. They were washed twice in the centrifuge with physiological saline. The bacterial paste contained about 20% dry material. 5 ml M/10 pH 7.0 phosphate buffer, 1 ml physiological saline and 0.85 ml of toluene were added to each gram of this paste. The suspension was preserved over night at 20°C. and used the next day for the estimation of hydrolase activity. Glass-ware and solutions were sterilized before use, since KLUYVER and CUSTERS (1940) observed, that even under toluene infection is possible.

5. Determination of hydrolase activity. An equal

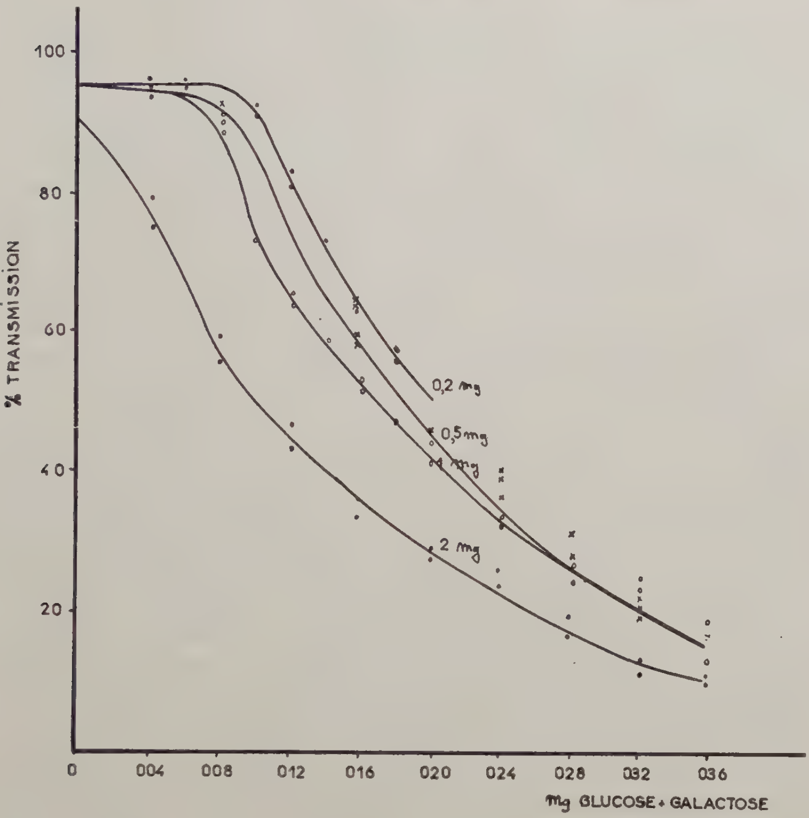

Fig. 1. Reference curve for the determination of the % hydrolysis of lactose by the method of Caputto, Leloir and Trucco. Each curve corresponds to a different weight of substrate (2, 1, 0.5 and 0.2 mg), initially present in the sample.

volume of a 2% solution of lactose or lactobionate was added to the suspension of autolyzed cells. A control without substrate was included. The mixtures were incubated in a water bath at 30°C. and samples of 0.2 ml or less were taken at regular intervals. The rate of monose formation was checked with the method described by Caputto, Leloir and Trucco (1947). Phosphates desensitize this method strongly but sodium and potassium had hardly any influence. The cell residues of *C. simplex* had no effect. The most appropriate phosphate concentration during the colour reaction was automatically obtained by using M/15 during the autolysis. The proteins readily precipitated by the addition of the copper salt, so that the solution had to be filtered previous to the colorimetric estimation. As the blue complex is adsorbed on cellulose fibres, it was of importance to chose the filter properly. The stability of the complex in regard to time is very satisfactory. After the reaction was completed at 80°C. and the arsenomolybdic reagent, sulfuric acid and water were added to a final volume of 10 ml, a pinch of kieselguhr was mixed with the blue turbid solution, which was then filtered twice on the same Green N° 402 filter. The transmission was read in reference to water in a nepho-colorimeter Coleman, filter N° 8–209. This method is very sensitive. When about 10% of the substrate was hydrolyzed, enough monosaccharides were produced to yield an intense blue colour, colorimetric estimation of which was just possible with a 0.2 ml sample. In the initial stages of the hydrolysis 0.2 ml sample were taken from the enzyme mixture, made up to 2 ml and the colour reaction performed. At a more advanced stage of the hydrolysis, 0.1 ml, 0.05 ml and still later 0.02 ml samples were taken. In order to keep the final phosphate concentration equal in all tests, the latter suspensions were made up with the reference suspension to 0.2 ml and then with water to 2 ml as usual. The transmission readings can be converted into the percentage of the hydrolyzed substrate by means of a series of reference curves, one for each initial concentration of substrate. These curves for lactose and lactobionate are represented in Fig. 1 and 2 respectively.

6. Chemical estimations.

Lactose in the growth medium was determined, after centrifugation, according to the Scales method, as amended by ISBELL, PIGMAN and FRUSH (1940).

Lactobionate was determined after hydrolysis for one hour at 100°C. in 0.75 N HCl, as described by KLUYVER, DE LEY and RIJVEN (1950)

7. Warburg experiments.

The conventional Warburg respirometer was used at 30°C. The main vessel contained 25 mg living cells and M/40 pH 7.0 phosphate buffer, the side arm contained 1,25 $\mu$moles lactose or lactobionate or 2,5 $\mu$moles glucose, galactose or gluconate.

## RESULTS.

### 1. Decomposition of lactose and lactobionate by growing cells.

*C. simplex* grows abundantly on a solid medium containing

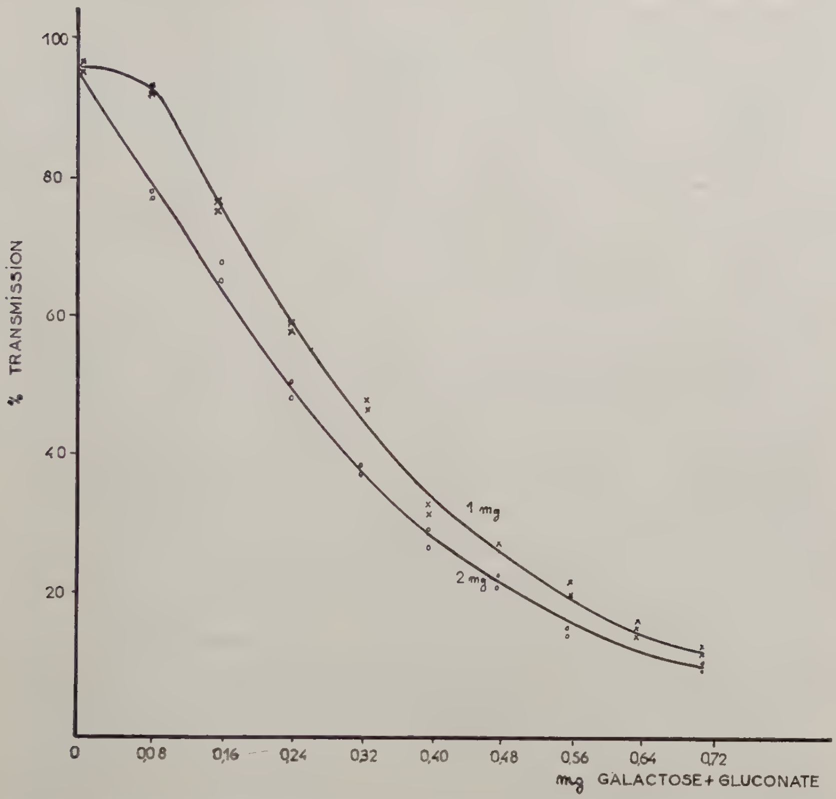

Fig. 2. Reference curve for the determination of the % hydrolysis of lactobionate by the method of CAPUTTO, LELOIR and TRUCCO. Each curve corresponds to a different weight of substrate (1 and 2 mg), initially present in the sample.

lactose or lactobionate as the main carbon source. Growth on liquid media is rather poor. No development was observed under anaerobic conditions. The results of an experiment, where *C. simplex* was grown on a liquid lactose or lactobionate medium, whilst agitated on a shaking device, is summarized in Table 1.

Lactobionate is consumed much faster than lactose, in spite of the apparently unfavourable course of the pH. Similar variations of the pH have been noted in the decomposition tests with other micro-organisms of our collection : the alcalinisation in the medium is always more conspicuous in the case of the bionates than with the corresponding disaccharides or monoses and is due to the cation which remains after the consumption of the organic anion.

TABLE 1.

Consumption of lactose or lactobionate, by growing *C. simplex*.

| Time | mg lactose/ml | pH | mg lactobionate/ml | pH |
|---|---|---|---|---|
| 0 | 34.3 | 6.7 | 28.4 | 6.8 |
| 24 h | 33.4 | 7.0 | 27.2 | 7.0 |
| 48 h | 28.9 | 6.7 | 17.1 | 7.6 |
| 72 h | 19.5 | 6.8 | 8.1 | 8.1 |
| 96 h | 16.6 | 7.0 | 1.1 | 8.25 |
| 120 h | 14.7 | 7.35 | 0 | 8.5 |

### 2. Comparison of the rate of lactose and lactiobionate consumption by resting cells with the activity of the β-galactosidase in autolyzed preparations.

In preliminary experiments it had been established that *C. simplex* possesses a very active hydrolytic enzyme, able to split lactose with the formation of monoses. The same crude autolyzed preparation was able to hydrolyze lactobionate, although at a slower rate, with the formation of a monose. An experiment was designed to check whether the activity of the hydrolytic enzymes of autolyzed preparations was sufficient to explain the rate of consumption of either substrate by an identical amount of resting cells.

Therefore cells were grown on either substrate, centrifuged and washed. The bacteria were suspended in phosphate buffer-saline as described in the experimental part. Toluene was added to one half and left overnight. The other half was preserved at 4°C. during the same length of time. The next day, the activity of the β-galactosidase for both substrates was determined in the autolyzed preparations. An identical amount of lactose or lactobionate was added to the resting cells. The latter suspensions were shaken in an incubator room at 30°C. and samples taken at regular intervals to determine the concentration of the remaining substrates. The results of one experiment are summarized in Fig. 3.

Autolyzed cells, grown on lactose, contain a very active hydrolase for this substrate. Cells grown on lactobionate contain likewise a hydrolase, which splits lactobionate with the formation of reducing substances, presumably galactose. The hydrolysis of lactose proceeds about 25 times as fast as the hydrolysis of lactobionate, which tallies with the data on the specificity of sweet almond β-galactosidase, reported by HELFERICH, PIGMAN and ISBELL (1939). However, the rate of consumption of both substrates by intact

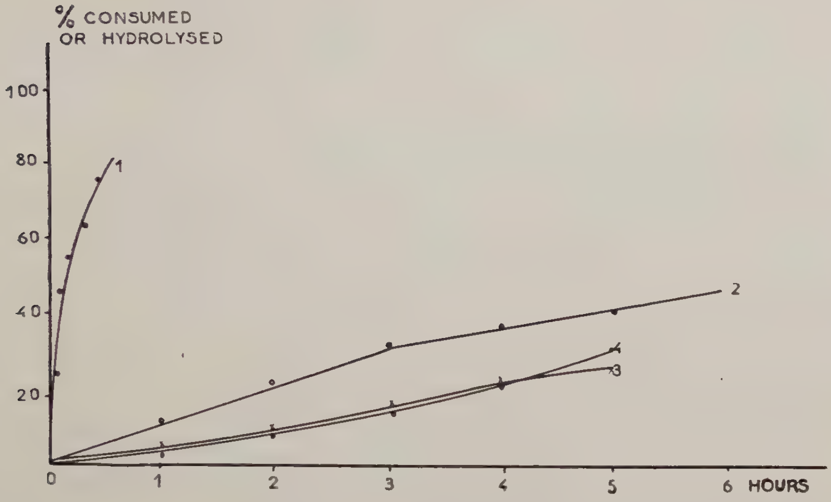

Fig. 3. Consumption of lactose (curve 4) and lactobionate (curve 3) by resting *C. simplex*, as compared with the hydrolysis of lactose (curve 1) and lactobionate (curve 2) by autolyzed cells under identical conditions.

cells is essentially identical and much slower than the rate of hydrolysis by the same amount of autolyzed cells under identical conditions. The activity of the hydrolases is thus greater than required for the consumption rate by the intact cell : lactobionate is hydrolyzed at about twice the rate in which it can be consumed and lactose at about 50 times the latter. The amount of hydrolases present is sufficient to account for the consumption.

### 3. **The oxidation of lactose and lactobionate by induced and uninduced cells.**

From the hypothesis, expressed in the literature, and from the previous paragraph one might be inclined to believe that the same enzyme is responsible for the hydrolysis of both substrates. The following experiments bring additional support for this conception. Uninduced cells were prepared by growing them on a glucose medium. The cells were also grown on a medium containing either lactobionate, lactose or lactose plus gluconate. Indeed, if one wants to check, whether the enzyme system from cells, grown on lactose, is also able to oxidize lactobionate without adaptation, allowance is to be made for a possible lack of adaptation to gluconate, a component of lactobionate. As has been shown by Cohen (1951)

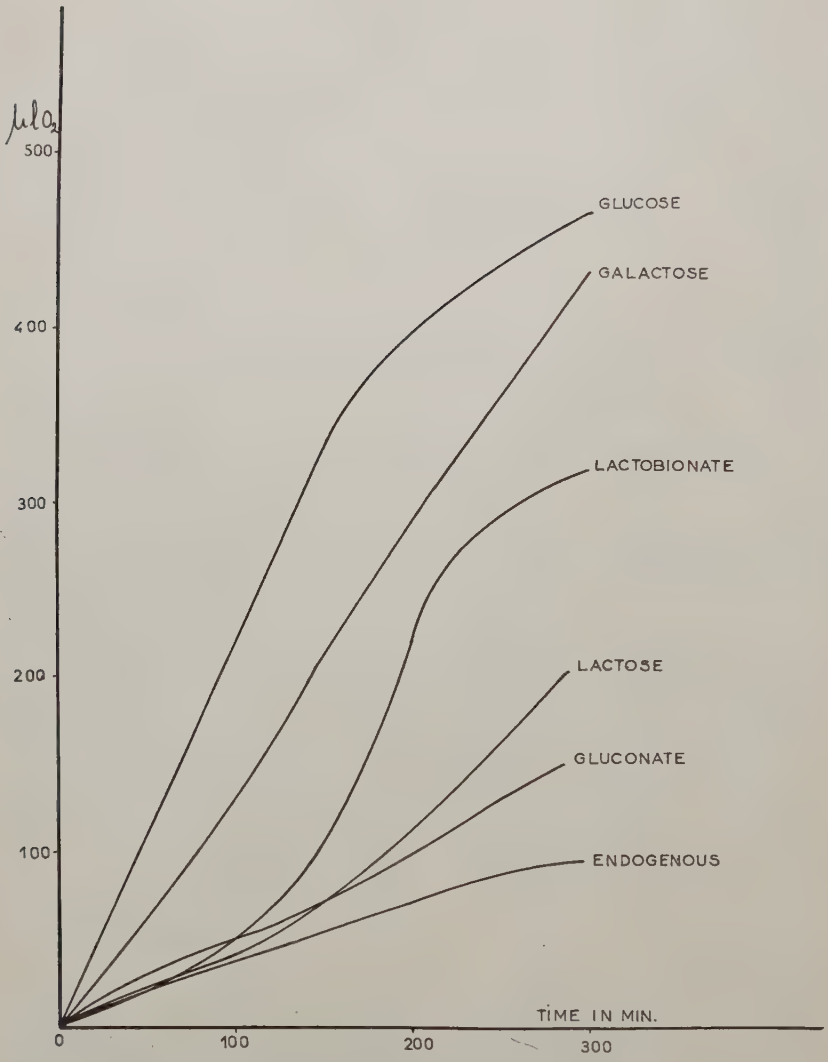

Fig. 4. Oxidation of several substrates by resting cells of glucose grown *C. simplex*.

and by DE LEY (1953) the consumption of gluconate by members of *Enterobacteriaceae* is initiated by an inducible kinase. The concentration of the gluconate in the culture medium is kept low (0.1%) in order to prevent preferential assimilation.

Resting cells from a glucose medium oxidize this substrate without an induction phase (Fig. 4). Galactose is oxidized after a

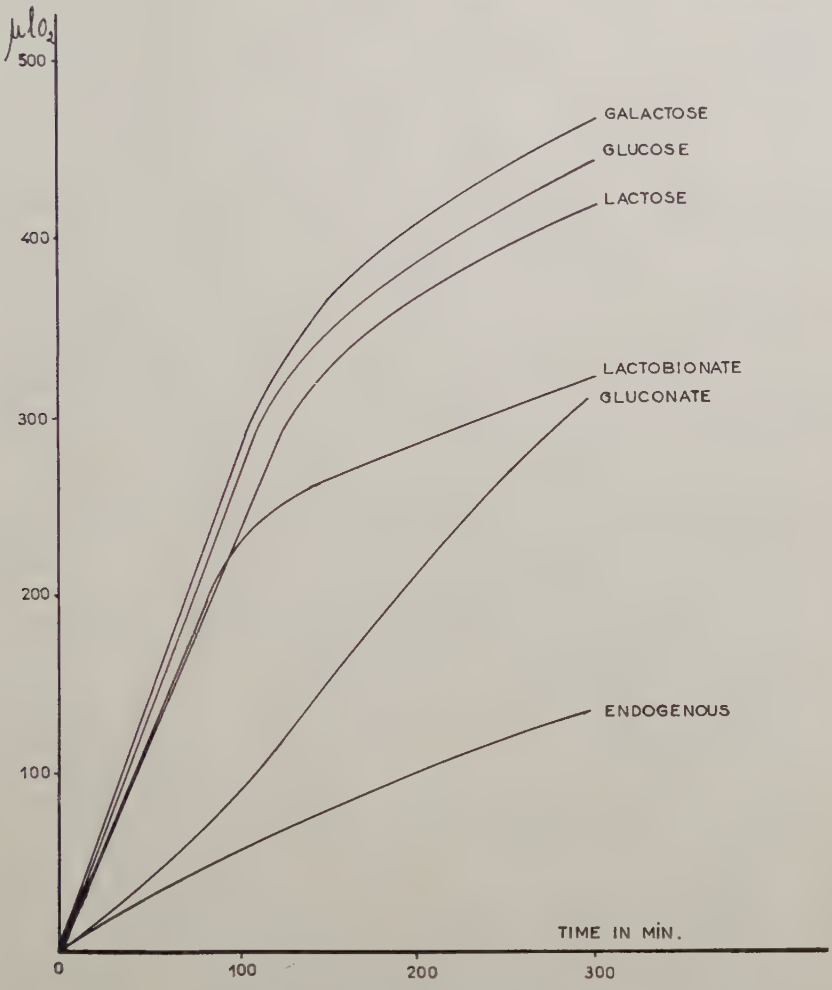

Fig. 5. Oxidation of several substrates by resting cells of lactose plus gluconate grown *C. simplex.*

slight, and the other substrates after a pronounced induction phase. This tends to prove that inducible enzymes are involved in the metabolism of these substrates. For galactose and gluconate, they are most likely the kinases, and for lactose and lactobionate, they are undoubtely the hydrolyzing enzyme. Cells grown on lactose plus gluconate (Fig. 5) oxidize galactose, glucose, lactose and lactobionate immediately. There is still a slight induction phase for the oxidation of gluconate which might be due to either an incomplete induction

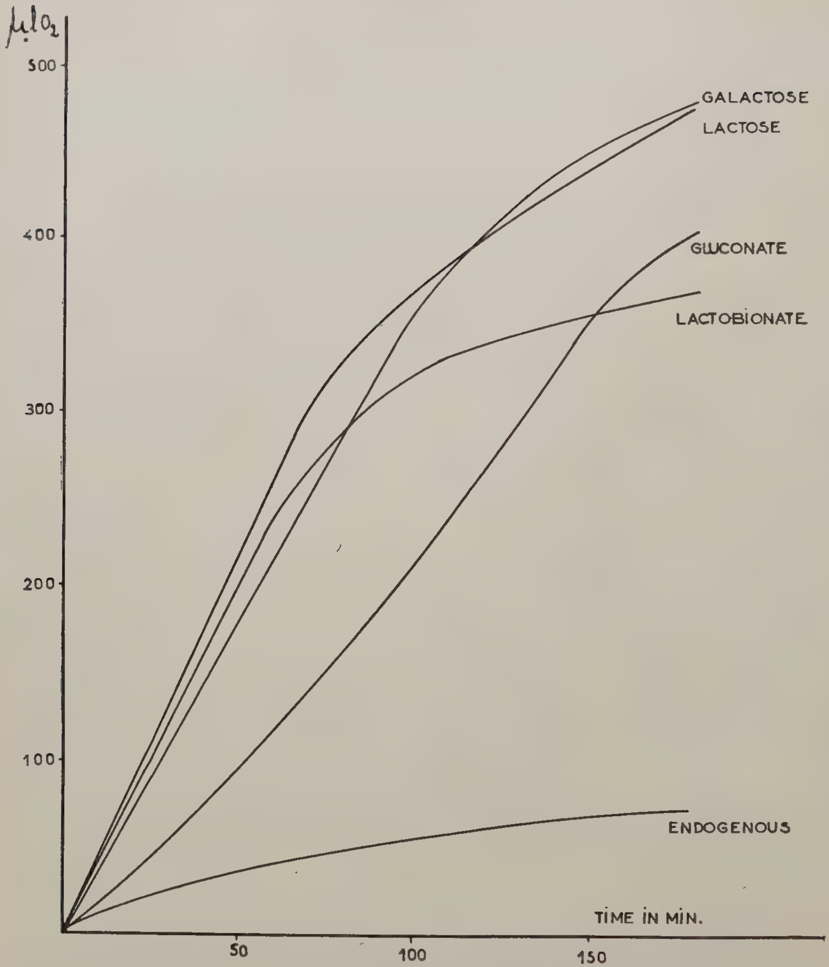

Fig. 6. Oxidation of several substrates by resting cells of lactobionate grown *C. simplex.*

during growth or to a deadaptation. Cells grown on lactobionate (Fig. 6) oxidize galactose, glucose, lactose and lactobionate directly. Again there is a slight induction period for gluconate. It is noteworthy that the induction phase for the gluconate respiration is much longer with glucose, than with lactobionate grown cells. The enzyme system for the oxidation of glucose is obviously constitutive. The cross-adaptation for lactose and lactobionate strengthens the idea that the same $\beta$-galactosidase is responsible for the metabolism of both substances. If they were indeed two different enzymes, cells, grown on lactose, would not contain the lactobionate-hydrolase and would show an induction period with the latter substrate, and vice versa.

## DISCUSSION and SUMMARY.

*Corynebacterium simplex* is able to use either lactose or lactobionate as a source of carbon for growth. Lactobionate is consumed much faster than lactose by growing cells. These cells, when grown on either substrate, contain an inducible $\beta$-galactosidase, which is not formed when grown on glucose. The rate of hydrolysis of either substrate by autolyzed cells amply suffices to account for the rate of their consumption by resting cells in identical conditions, so that no additional mechanism has to be postulated. Some other step in the metabolism of both substances is the limiting factor in the consumption rate. It seems most likely for the moment that permeability phenomena interfere with the consumption of the substrates by intact cells since no accumulation of monosaccharides was observed during the oxidation experiments, although this could be expected on account of the strong lactase activity. Cells grown on lactose plus gluconate oxidize lactobionate without an induction phase; likewise, lactobionate grown cells oxidize lactose at once. Glucose grown cells use both substances as respiration substrates only after a pronounced induction phase. This cross-adaptation indicates that it is the same enzyme which is responsible for the hydrolysis of both substrates. The behaviour is in agreement with the theory of WEIDENHAGEN, as modified by PIGMAN, on the specificity of glycosidases.

The remaining steps in the metabolism of both substances can now easily be inferred from the knowledge of the intermediary metabolism in other species of the same genus. Glucose, gluconate and galactose will probably be phosphorylated by a kinase and the phosphoric esters metabolized further by way of the hexose monophosphate oxidative cycle (shunt) and possibly the Krebs cycle; the former two substances might also undergo a "direct hexose oxidation" (GHIRETTI and GUZMAN BARRON (1954), JÄNNES (1954), ZAJIC, DE LEY and STARR (1956)).

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# CORRELATION BETWEEN HEXOSE-MONOPHOSPHATE SHUNT, GLYCOLYTIC SYSTEM AND FERMENTATION-TYPE IN LACTOBACILLI[2]

by

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(Received July 20, 1957).

## INTRODUCTION.

GIBBS *et al.* (1955) discovered that the heterofermentative species *Leuconostoc mesenteroides* did not possess the glycolytic enzyme aldolase. In a previous publication one of us (BUYZE 1955) showed that *Lactobacillus brevis*, another heterofermentative organism, also lacks aldolase. On the basis of fermentation balances and aldolase estimations it was suggested at that time that hexose monophosphate oxydation and fermentation of the resulting pentoses (H. M. P. shunt[1]) would be the major pathway of carbohydrate breakdown in heterofermentative Lactobacilli, whereas in the homofermentative lactic acid bacteria the glycolytic system would be of prime importance. Moreover our results indicated that a mixed fermentation type, possessing and using both systems simultaneously, could exist. More recently GIBBS *et al.* (1955) with the aid of $C^{14}$ labelled glucose showed that heterofermentative lactic acid bacteria metabolized glucose along the H.M.P. shunt.

The preliminary results of the determination of some of the key enzymes of both pathways of glucose breakdown in cel-extracts of various Lactobacilli are reported in this paper.

## MATERIALS AND METHODS.

The used **substrates** were obtained commercially. 6–P–G[1]) was prepared from G–6–P[1]) by oxydation with bromine (SEEGMILLER *et al.* 1951). F–1–6–P[1]) was prepared from sucrose (NEUBERG 1942).

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[1] Abbreviations. H.M.P. shunt = hexose monophosphate shunt. G–6–P = glucose–6–phosphate. 6–P–G = 6–phosphogluconic acid. F–1–6–P = fructose–1,6–diphosphate.

[2] This investigation was supported by a grant of the Neth. Org. for Pure Research (Z.W.O.).

The Na-salts were prepared from the Ca- and Ba-salts by treatment with Dowex 50 and neutralization with sodiumhydroxide.

**Media.** Our basic medium is a modification of the medium described by Rogosa *et al.* (1951). It contained in grams per l: trypticase (BBL) 5, yeast extract 5, $KH_2PO_4$ 3, ammoniumcitrate 1, salt solution (Rogosa l.c.) 2.5, Tween 80 0.5, sodium acetate 15. The medium was sterilized by autoclaving. After sterilization carbohydrate (glucose or gluconate) was added to a final concentration of 1%. The pH was 6.8.

For the culture of *L. bifidus* 0.05% ascorbic acid was added. *L.brevis* was cultured in the synthetic medium described by Buyze (1955). *L. lactis* and *L. delbrückii* were cultured in the same medium enriched with 10% tryptic casein hydrolysate. *L. bulgaricus* was cultured in the medium described by Wright *et al.* (1950), enriched with 2% v/v of a 10% yeast autolysate as a source of orotic acid.

The bacteria were cultured in 500 ml quantities in 1 l flasks at a temperature of 37° C. except *Leuc. mesenteroides* and *Leuc. dextranicus*, which were cultured at 30° C. The gaseous phase was air, except in the case of *L. bifidus* which was cultured under nitrogen with 5% carbon dioxide.

In the used media growth is poor if the carbohydrate is omitted. By culturing in tubes under liquid paraffin it was possible to determine whether a carbohydrate can be used as a sole carbon source under anaerobic conditions.

**Cell free extracts.** Washed bacterial cells were suspended in saline or in 0.1% sodium acetate. Suspensions were standardized by turbidity and contained about 60 mg (dry weight) cells per ml. Cell-free extracts were prepared in a Mickle desintegrator with 5.5 g ballotini no 11 per 5 ml cell-suspension. After desintegration the extract was centrifuged 30' at 1500 g.

**Aldolase** was estimated according to Sibley and Lehninger (1949).

**G–6–P–dehydrogenase** and **6–P–G–dehydrogenase** were determined by estimating the reduction of TPN with an extinctometer at $\lambda = 366$ m$\mu$.

Mixtures contained 0.27 $\mu$ mol TPN (or DPN), 125 $\mu$ mol glycyl-glycine-sodium hydroxide buffer at pH 7.6, 50 $\mu$ mol $MgCl_2$ in 4.6 ml $H_2O$. Cellextracts were added in 0.2 ml quantities. The extinction was then estimated and 0.5 $\mu$ mol substrate in 0.2 ml was added. The increase of extinction was plotted against time.

### FERMENTATION TYPE.

Homofermentation and heterofermentation are taken here to refer only to the metabolic products produced from glucose as a substrate.

The fermentation type was studied by culturing with a Durham tube under liquid paraffin according to DAVIS (1955). Gas formation was noted daily for four days. The fermentation type was further established by Warburg experiments and fermentation balances.

All results were in accordance with the literature (Bergey 1948) with the exception of *Leuconostoc citrovorum*. Both strains of *Leuconostoc citrovorum* were found to be homofermentative. It has been claimed that *Leuconostoc citrovorum* 8081 should be classified as a (homofermentative) *Pediococcus* (FELTON *et al.* 1953). The present results suggest that the taxonomic status of *Leuc. citrovorum* 8082 should also be considered with some doubt.

### RESULTS AND DISCUSSION.

The results of the experiments are compiled in table 1. All strains grew with glucose as sole carbon source; only four produced gas from glucose anaerobically (col. 2.) All homofermenters (except *L. bifidus*) produced only lactic acid from glucose (col. 3). All the homofermenters contain the enzyme aldolase (col. 4), whilst this enzyme is absent in the heterofermenters. The heterofermenters always contain G–6–P– and 6–P–G–dehydrogenases. These results seem in accordance with the hypothesis described in the introduction.

On the other hand columns 5 and 6 show that many of the homofermenters possess the dehydrogenases of the oxydative pathway. Though these enzymes are present, only lactic acid and no $CO_2$ is produced from glucose. We suggest that these enzymes are not used for glucose dissimilation under the conditions of our experiment. The formation of two moles of lactic acid from one mole of glucose via 6–phospho–gluconic acid is of course theoretically possible, but such a supposition is not supported by the isotope experiments of GIBBS *et al.* (1955) with *Leuc. mesenteroides*. It is of course possible that the presence of the dehydrogenases in homofermentative Lactobacilli means that these organisms under other circumstances metabolize glucose along the oxydative pathway. Comparable phenomena have been observed with *Bac. subtilis* (GARY *et al.* 1952, 1954), *E. coli* (COHEN 1951; COHEN and ROTH 1953) and many others. For *L. casei* we have some indications that this is the case. The possibility that these enzymes are not important for glucose

TABLE 1.

| | | gas from glucose | products from glucose | aldo-lase 1) | G-6-P-dehydr. | | 6-P-G-dehydr. | | growth on GA | gas from GA |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | TPN | DPN | TPN | DPN | | |
| **Homofermentative strains:** | | | | | | | | | | |
| *L. delbruckii* | | — | 2 la | + | — | — | — | — | — | — |
| *L. lactis* | LL 4 | — | nd | + | — | — | — | — | — | — |
| *L. leichmanni* | ATCC 4797 | — | 2 la | + | — | — | — | — | — | — |
| *L. bifidus* | isol. | — | la + ac | + | — | | ± | | — | — |
| *L. plantarum* | Orla Jensen | — | nd | + | + | — | + | — | — | — |
| *L. bulgaricus* | ATCC 8001 | — | 2 la | + | + | | ± | | — | — |
| *L. arabinosus* | ATCC 8014 | — | 2 la | + | + | — | ++ | — | — | — |
| *L. parabifidus* | TH Delft | — | 2 la | + | +++ | ± | + | ± | — | — |
| *Leuc. citrovorum* | ATCC 8081 | — | 2 la | + | ++ | — | + | — | — | — |
| *Leuc. citrovorum* | ATCC 8082 | — | 2 la | + | +++ | — | ++ | — | + | + |
| *L. casei* | ATCC 7469 | — | 2 la | + | + | — | ++ | — | + | + |
| *Str. faecalis* | isol. | — | 2 la | + | ++++ | — | +++ | ± | + | + |
| *Str. lactis* | NTCC 2700 | — | 2 la | + | ++ | — | + | — | + | + |
| **Heterofermentative strains:** | | | | | | | | | | |
| *L. brevis* | TH 523 | + | 1 la + 1 ac + 1 $CO_2$ 2) | — | +++ | — | + | — | + | + |
| *L. fermenti* | TH Delft | + | 1 la + 1 $CO_2$ 3) | — | ++ | — | + | — | + | + |
| *Leuc. dextranicum* | ATCC 8086 | + | 1 $CO_2$ 3) | — | +++ | + | ++ | ± | — | — |
| *Leuc. mesenteroides* | ATCC 8293 | + | 1 la + 1 $CO_2$ 3) | — | +++ | —4) | ++ | — | — | — |

la = lactic acid  ac = acetic acid  2 la = 2 moles of lactic acid per mole glucose  nd = not determined  GA = gluconic acid.

1) Though differences in the aldolase-activity of various extracts were observed, insufficient data were available to warrant other conclusions than the presence or absence of the enzyme.

2) In the presence of a hydrogen acceptor: 1 glucose + 2 Acc → 1 la + 1 ac + $CO_2$ + 2 $AccH_2$ (comp. Buyze 1955).

3) Further products (acetic acid and ethanol) not determined.

4) De Moss observed that the dehydrogenase of his *Leuconostoc* could reduce DPN. He used another strain however.

dissimilation at all but solely provide metabolites for assimilative purposes cannot be excluded [1]).

Some of the homofermenters with both dehydrogenases are able to use gluconate as sole carbon source (col. 7). If they do they always produce $CO_2$, hereby proving their heterofermentative potency (col. 8). Those who do not use gluconate presumably lack gluconokinase.

*L. bifidus* is a homofermenter in the sense that it does not produce gas from glucose, but might be considered a heterofermenter as it produces acetic acid and lactic acid. The formation of acetic acid whilst G–6–P–dehydrogenase is absent and the fact that *L. bifidus* can fix $CO_2$ (KUHN *et al.* 1953, PINE 1956) seem to indicate that the metabolism of this organism is extraordinary, which is of course in accordance with the peculiar taxonomic position of *L. bifidus*.

The presence of a few enzymes of a metabolic pathway does not of course prove the presence of a complete system. Pending further work it seems however that the *Lactobacteriaceae* can be divided in a small group of obligate homofermenters lacking G–6–P– and 6–P–G–dehydrogenase, a small group of obligate heterofermenters lacking aldolase and a fairly large group, which though possessing the glycolytic and the oxidative pathway, behave generally as homofermenters. Whether this last group can be described as facultative homofermenters and under which conditions the H.M.P. shunt is used is under study.

It is obvious that more sensitive methods of enzyme determinations, or different ways in preparing cell-free extracts might detect traces of aldolase in some obligate heterofermenters or of the dehydrogenases in some obligate homofermenters. This does not invalidate the general trend. On the contrary, the existence of the large intermediate group stresses the interrelation between the patterns of glucose dissimilation in *Lactobacteriaceae*, which probably evolved from a few orginal types.

## Summary.

The presence of the enzymes glucose–6–phosphate–dehydrogenase, 6–phosphogluconic acid-dehydrogenase and aldolase was studied in

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[1]) Experiments on oxygen consumption with glucose as a substrate showed that glucose is metabolized along the oxydative pathway by all strains containing the dehydrogenases. The amount of $O_2$ consumed was very low however for the strains with low dehydrogenase activity. In the presence of oxygen both pathways are indeed functional in the strains that posses both systems.

a number of *Lactobacteriaceae.* Heterofermenters always lacked aldolase. The homofermenters could be divided in two groups according to the presence or absence of the dehydrogenases of the oxydative pathway of glucose breakdown. It is suggested that the *Lactobacteriaceae* can be divided in three groups:

a. obligate heterofermenters lacking aldolase;
b. obligate homofermenters lacking the dehydrogenases;
c. facultative homofermenters, which contain both dehydrogenases and generally metabolize glucose along the glycolytic pathway.

## Acknowledgement.

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# A THERMOPHILIC, MOTILE STRAIN OF *MICROBACTERIUM*

by

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## INTRODUCTION.

In a recent brief note VAŇKOVÁ (1957) has referred to motile catalase-producing bacteria similar in their characteristics to *Thermobacterium cereale* Orla-Jensen (1942) and considered in Bergey's Manual (1948) to be a synonym for *L. delbrückii*. Also DACRE and SHARPE (1956) have isolated, from cheddar cheese, typical strains of *L. plantarum*, which possess a strong catalase activity.

In the present paper we report the characteristics and some biological properties of a new homofermentative lactic acid bacterium, which is distinguished from the genus *Lactobacillus* by its motility and by its catalase content, a strain probably similar to those isolated by VAŇKOVÁ (1957).

## METHODS.

The characters of our microorganism have been determined, unless otherwise stated, by the procedures described in the Manual of Pure Culture Study issued by the Committee on Bacteriological Technic of the Society of American Bacteriologists (1948). For the purpose of convenience and completeness, all media used are listed below:

Nutrient broth: peptone, 5 g; beef extract, 3 g; distilled water, 1000 ml; pH 6.5.

Saccharose broth agar: nutrient broth with 1 per cent saccharose.

Nutrient agar: nutrient broth with 1.5 per cent agar.

Saccharose agar: nutrient agar with 1 per cent saccharose.

RESULTS AND DESCRIPTION.

Habitat: Vegetables, grain mashes and probably soil.

Morphology: Rods 0.7 by 3 to 6 $\mu$, occurring singly and in short chains with rounded ends. Under adverse conditions they tend to be longer specially as the acidity of the fermenting vegetables increases. No capsules and endospores were formed. Gram-positive and non-acid-fast.

Motility: Motility was observed in all substrates tested: nutrient and saccharose broth, semi-solid agar, beet juice and beet molasses; the best motility was obtained in the first hours of growth and disappeared within 24 hrs. Staining the long flagella by the method of LEIFSON (1951) peritrich forms were observed.

Macroscopic growth: Growth on saccharose agar or nutrient agar slants at 37°C. was effuse in 24 hrs but became heavier in 2 to 3 days. Colonies on the same substrates were circular, raised, smooth, opaque, with entire edge, about 3 to 3.5 mm in diameter. The line of puncture was beaded. Growth in 24 hrs on saccharose broth cultures was diffuse at 52°C. with a compact and abundant sediment. Particular odor absent. Growth on nutrient broth was good, while growth was scant in the following synthetic medium: sucrose 10 g; $KH_2PO_4$ 1.5 g; $Na_2HPO_4 . 2H_20$ 5 g; $MgSO_4 . 7H_2O$ 0.2 g; $(NH_4)_2SO_4$ 3 g; distilled water 1000 ml; pH 6.5. More rapid and abundant growth was obtained from aerated cultures.

Hydrolysis of gelatin. The cultures were streaked once across duplicate plates of saccharose agar plus 0.4 per cent gelatin and incubated at 35°C. One plate was for estimation of hydrolysis of the gelatin at 5 days and the second at 10 days by flooding with 10 ml of the FRAZIER (1926) solution. No liquefaction of the gelatin occurred at the first culture, but after four transfers, on the same substrate, liquefaction was clearly visible. Consequently, this organism is able to hydrolyse the gelatin by adaptation.

Temperature relations. Optimum growth in saccharose broth occurred at 50°C., while it was scant at 80°C. and did not occur at 10°C. after 20 days of incubation. The organism survived 85°C. for 10 minutes and the thermal death point was found at 90°C. to be 2 minutes.

Optimum of pH for growth. 12 tubes containing 10 ml of sterile saccharose broth to a pH of 4.5 to 9.0 were inoculated with some drops of a fresh culture of the organism. Upon incubation at 50°C. for 10 and 24 hrs, the tubes were examined for growth by direct microscopic observation and by determination of the final pH. The pH optimum for growth was from 6 to 7 and the limits for growth from pH 5 to 8.5. Final pH 4.5.

Relation to free oxygen. A tube of deep saccharose agar was inoculated while in fluid condition at 45°C. with an inoculum not too heavy to permit discrete colonies, rotated to mix the inoculum with the medium and cooled. After incubation at 35°C. growth was observed throughout the medium but was heavier upon the surface and in the upper layers.

The organism can grow under anaerobic condition but the best growth occurs in aerated cultures by bubbling air or oxygen in the medium. Therefore, the strain can be considered aerobic to facultative anareobic.

Catalase formation. Formation of catalase was observed by visible reaction with hydrogen peroxide, i.e. by flooding with 10 ml of a 3 per cent hydrogen peroxide solution saccharose agar slant colonies. However, because of the importance of catalase formation in this organism a need for more quantitative evidence seemed to be indicated. Washed cells from 48 hrs saccharose agar cultures were suspended in 0.006 M phosphate buffer (the density determined optically) and their ability to decompose hydrogen peroxide measured in a Warburg manometer by observing the rate of oxygen liberation (FUJITA and KODAMA, 1931). The catalase quotient has been found about 1.900 at pH 7 and at the incubation temperature 37°C. VAŇKOVÁ (1957) has found a value of 2.000 for her strains.

Acidity in milk. 10 ml of skim-milk were sterilized in 15 by 180 mm tubes, inoculated by loop and incubated for a week at 37°C. The contents of the tubes were washed into Erlenmeyer flasks, titrated with N/10 NaOH and the acidity value corrected for control acidity. No acidification of the milk was observed.

Rennet production. Rennet formation was clearly visible using the method of GORINI (1932).

Acid production from carbohydrates. 0.1 g of the pure carbohydrates to be tested was dissolved in 10 ml of nutrient broth placed in Durham's fermentation tubes. These were sterilized in the Arnold by the intermittent method for three successive days and inoculated with three loops of a fresh culture of the organism. A first series of tubes was incubated at 37°C. and a second series at 50°C. for 7 days. Care was taken to avoid evaporation. At the end of the incubation period growth was determined measuring the optical density and the pH of the medium in each tube. Growth and acidification, without formation of gas, were observed for the following substrates: glucose, fructose, mannose, sucrose, maltose, melizitose, raffinose, trehalose and dextrin. No growth occurred in presence of arabinose, xylose, rhamnose, galactose, lactose, inulin, starch, glycerol, erythritol, mannitol, sorbitol, dulcitol and salicin.

Utilization of citrate. No utilization of citrate was observed both in Koser and in the mineral medium previously reported (see macroscopic growth).

Reduction of nitrate to nitrite. All cultures were negative.

NaCl tolerance. The culture was inoculated into 4 tubes of saccharose broth containing 0, 1, 2.5, and 5 per cent of NaCl, respectively. Care was taken to avoid transfer of a large amount of inoculum. The growth in the 4 tubes was compared after 48 hrs incubation at 50°C. and found positive in all tubes with a slight retard in 5 per cent NaCl.

Indole production. All cultures were negative.

Lactic acid production. No volatile acids were detected in steam distillates of the fermented saccharose nutrient broth. Slight acidity of the distillate was found to be due to traces of lactic acid that were carried over during distillation. Lactic acid was the only acid found in the fermented medium. The specific rotation and the water content of the zinc lactate, cristallized from the ether extract of the medium, demonstrate that the lactic acid produced was for the most part inactive.

## DISCUSSION.

The fermentation reaction and morphology of the organism described indicate that it should be classified as a member of the family *Lactobacteriaceae.* In placing an unknown organism in this family as it is defined in Bergey's Manual (1948), rods fermenting carbohydrates to produce mainly lactic acid must fall in either the genus *Lactobacillus* or *Microbacterium.* These genera are separated entirely on the basis of catalase production by members of the latter but not of the former.

The unknown organism described here seems to belong to the genus *Microbacterium* by reason of its catalase production and for its high heat resistance, although by its morphology, by some secondary characters and its fermentative properties it resembles more nearly the members of the genus *Lactobacillus*. Contrary to the characteristics commonly ascribed to the genus *Microbacterium* our organism is motile. But motility represents frequently an exception for some genera, for instance for the genus *Lactobacillus* for which Harrison and Hansen (1950), Mann and Oxford (1954) and Cunningham and Smith (quoted from Tittsler *et al.*, 1952) have described motile strains. Therefore it cannot be excluded that also in the genus *Microbacterium* there are motile strains. McLean and Sulzbacher (1953) have found that lactic acid was the only end product in the fermentation of *Microbacterium thermosphactum* in accord with the results obtained with our organism.

Placing in the genus *Lactobacillus* this organism and probably the catalase producing strains of Vaňková (1957) and those of Dacre and Sharpe (1956) would involve abolishment of the genus *Microbacterium*, the "catalase production" being the main distinctive character separating this genus from the former. For this reason we have preferred to place our organism in the genus *Microbacterium*. The name *Microbacterium mobile* is proposed.

## Summary.

A thermophilic, motile organism producing catalase is described. It belongs certainly to the genus *Microbacterium* by reason of its catalase production, although in its morphology, in some secondary characters and in its fermentative properties it more nearly resembles members of the genus *Lactobacillus*. The name *Microbacterium mobile* is proposed for this organism.

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# ANTIMICROBIAL SUSCEPTIBILITY AND SOME OTHER PROPERTIES OF PHOTOCHROMOGENIC MYCOBACTERIA ASSOCIATED WITH PULMONARY DISEASE

by

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## 1. INTRODUCTION.

Mycobacteria capable of producing luxuriant pigment have up to a few years ago nearly invariably been looked upon as saprophytes. Recent reports (4, 6, 8, 13, 15–19, 22–25) make it highly probable that at least some of these chromogenic species may be regarded as pathogens owing to the fact that they may be found in close association with pulmonary diseases strongly resembling tuberculosis. Infections probably due to atypical chromogenic mycobacteria have earlier been described in this country by DINGER (7) and by BEKKER (2,3).

We recently noticed three cases in patients in which roentgenographic and bacterioscopic findings evidenced pulmonary tuberculosis, as a result of which all of them had been admitted to hospitals ("Zonnegloren" sanatorium, Soest, and "Zonnestraal" hospital, Hilversum).

Specimens of sputum of the three patients demonstrated on smear acid-fast bacteria in a density of about +1 to +3 (Bronkhorst scales) on several occasions. On culture, within 4 to 6 weeks all specimens grew colonies (from ten to many hundreds per tube of Löwenstein medium) which, on first detection, were only difficultly distinguishable from those of *Myc. tuberculosis*. On exposure to daylight at room temperature the colonies developed a bright lemon-yellow pigment within a couple of days, indicating that the growth was not that of the type of organism being responsible for normal tuberculosis.

The organisms recovered from all three patients could be identified as so-called "yellow bacilli" (*Mycobacterium tuberculosis luciflavum* according to MIDDLEBROOK (14)) on account of the following properties: production of a characteristic photochromogenic pigment, typical aspect and texture of colonies (smooth and easily emulsified), negative neutral-red reaction, strong catalase activity, poor cord formation, and typical appearence of cells which were longer and thicker and often more beaded in the Ziehl-Neelsen stain than those of the normal tuberculosis organism.

The photochromogenic organism being practically non-pathogenic to a number of experimental animals, including guinea pigs (13, 15, 16, 17, 19, 25), the possibility of a co-existent true tuberculosis could be ruled out by an animal test. In fact, the three patients harbouring the atypical bacteria were all free of guinea pig virulent organisms as was established in two animal tests for each patient. Since none of the patients had received I.N.H., which may affect the virulence of *Myc. tuberculosis* (11, 20, 21) and thereby interfere with the diagnosis of tuberculosis, we were apparently confronted with the probability of a true infection of the patients with a hitherto poorly defined organism.

## 2. COMPARISON OF IN VITRO SUSCEPTIBILITY TO DRUGS OF PHOTOCHROMOGENIC BACTERIA AND OTHER MYCOBACTERIA.

Many mycobacteria, especially the group of acid-fast saprophytes, have up to now defied accurate classification. On the other hand, the organisms causing human, bovine and avian tuberculosis are well-defined species. The place of the photochromogenic organism among the mycobacteria is still uncertain.

An important factor in the differentiation and classification of mycobacteria is the relative susceptibility to the drugs isonicotinic acid hydrazide (I.N.H.), p-aminosalicylic acid and the thiosemicarbazones (1, 5, 9, 10). In this study accurate determination of sensitivity employing relative large numbers of concentrations was endeavoured to detect also the minor differences.

Drug sensitivity determinations were carried out using as a medium Herrold egg yolk agar slants and as an inoculum 1 to 2 drops of a suspension in 5 ml saline of one loop full of the growth of the organisms on Loewenstein slopes. To effect a homogeneous inoculation the suspensions were prepared by vigorously shaking the colonies, or fragments thereof, with glass beads. The substances examined were employed in the concentrations 0–0.1–0.2–0.5–1–2–5–10–20–50 and 100 μg per ml of the solid medium. Where not otherwise recorded, readings were taken after 2 weeks of incubation at 37°C.

The following strains have been compared: *Myc. tuberculosis* var. *hominis* (strain H 37 Rv); *Myc. tuberculosis* var. *bovis* (strain D 114); *Myc. avium* (strain used for the production of avian tuberculin); *Myc. phlei* (strain Delft); *Myc.* spec. (an acid-fast saprophyte isolated from gastric lavage); and the three strains of photochromogenic mycobacteria (strain L., strain Vr., and strain Tr.).

The result of the susceptibility determinations are summarized in table 1.

TABLE 1.

Drug sensitivity of various mycobacteria, recorded as minimum inhibitory concentrations in μg per ml of medium.

| Organisms / Substances | *M. tuberculosis* H37Rv | *M. tuberculosis* D 114 | *M. avium* | Photochromogenic strain "L." | Photochromogenic strain "de Vr." | Photochromogenic strain "Tr." | *M. phlei* | *M.* spec. (acid-fast saprophyte) |
|---|---|---|---|---|---|---|---|---|
| isonicotinic acid hydrazide | 0.1 | 0.1 | 1–2+) | 1 | 1 | 1 | >100 | >100 |
| p-aminosalicylic acid | 0.5 | 0.5 | 20 | 20 | 20 | 10-20 | >100 | >100 |
| thiosemicarbazone | 0.2 | 0.5 | 1 | 1 | 1 | 1++) | >100 | >100 |
| streptomycin | 1-2 | 1-2 | 2 | 2 | 2 | 1-2 | 2 | 2 |
| cycloserin | 10 | 20 | 10 | 10 | 10 | 20 | 20 | 20 |
| viomycin | 10 | 10 | 10 | 10 | 5 | 5-10 | 20 | 10 |
| tetracyclin+++) | 10 | 5 | 100 | 20 | 20 | 50 | 5 | 5 |

+) sensitivity read after 7 days of incubation.
++) reversal effect (zone of partial growth) at conc. 2-10 μg/ml.
+++) sensitivity read after 10 days of incubation.

It appears from the table that the different species do not display much variation with regard to sensitivity towards the antibiotics streptomycin, viomycin and cycloserin. The reaction to tetracyclin shows some more variation; as this drug is amenable to deterioration, particularly on long incubation, no pertinent conclusions can be drawn, however.

The susceptibility of the different organisms to I.N.H., p-aminosalicylic acid, and thiosemicarbazone shows a more definite pattern. The three strains of photochromogenic organisms appear to be easily differentiated from *Myc. tuberculosis* and saprophytic mycobacteria by virtue of their sensitivity to 1 μg/ml of I.N.H. and to about 20 μ/ml of p-aminosalicylic acid. It will be noted that these figures are nearly exactly intermediate between those of the organisms causing tuberculosis of either the human or bovine type and those of the saprophytic species. Obviously, in these respects the three photochromogenic organisms are intercalary between the organisms possessing pronounced parasitic properties and the true saprophytic mycobacteria.

With regard to sensitivity towards I.N.H., p-aminosalicylic acid, and thiosemicarbazone the photochromogenic strains are strikingly similar to *Myc. avium*. There is, nevertheless, no identity with the latter, which follows from the result of some observations on the catalase activity of the strains concerned. In the presence of 15% $H_2O_2$ and 5% Tween 80 in water (12) the avian strain was found to produce oxygen with about the same intensity as that normally observed in cultures of tubercle bacteria of human or bovine origin (5, 10). The three photochromogenic strains, on the other hand, had the abundant catalase activity characteristic of most strains of saprophytic mycobacteria (5). We further noticed that the isolated colonies which appeared on further incubation of tubes containing 10 μg I.N.H. per ml of medium fully retained their high catalase activity in the case of the photochromogenic strains, whereas with *Myc. avium* and *Myc. tuberculosis* this activity was completely lost.

In vitro, the main distinctive properties of *Myc. tuberculosis*, *Myc. avium*, a photochromogenic organism, and a typical saprophytic *Mycobacterium* appear to be as given in table 2.

## 3. DISCUSSION.

There are a number of properties which set the three strains of photochromogenic mycobacteria studied herein apart from all other mycobacteria. As mentioned previously, unique features of this type of organisms are the production of a light-conditioned lemon-yellow pigment, the stout cell habitus, the strong catalase activity which is fully maintained in the mutants possessing a high degree of I.N.H. resistance, and the lack of pathogenicity for the

TABLE 2.

Comparison of main in vitro properties of photochromogenic mycobacteria with other groups.

| | *Myc. tuberculosis* | *Myc. avium* | Photochromogenic mycobacterium | Saprophytic *Mycobacterium* |
|---|---|---|---|---|
| sensitivity to I.N.H. | ++ | + | + | — |
| sensitivity to P.A.S. | ++ | + | + | — |
| sensitivity to thiosemicarbazone | ++ | ++ | ++ | — |
| catalase activity of parent strains | + | + | ++ | ++ |
| ibid. of I.N.H. resistant strains | — | — | ++ | ++ |
| pigment | — | ++ | ++ | ++ |
| growth at 24°C. | — | — | —*) | ++ |
| neutral-red reaction | ++ | — | — | — |

++ = strong
+ = medium
— = absent or small

*) = growth at 24°C. may commence after some transfers in vitro.

guinea pig and a number of other laboratory animals including fowl.

Photochromogenic mycobacteria have several points in common with avian mycobacteria, the most important of which appear to be the great similarity in susceptibility to I.N.H., p-aminosalicylic acid and thiosemicarbazone, and the result of the neutral-red reaction which we found to be negative for both types of organisms. It is interesting to note that already in 1934 DINGER (7) reported on the similarity noticed between the strain of atypical mycobacteria studied by him and the organism responsible for tuberculosis in fowl.

As all three strains of photochromogenic organisms described herein were identical and remarkably constant in their properties, it would appear that in these organisms we are confronted with a new taxonomic entity. MIDDLEBROOK (14) has proposed as a name for the photochromogenic organism *Mycobacterium tuberculosis luciflavum*; as this species differs from *Myc. tuberculosis* in more than one important respect (e.g. pathogenicity to animals, pigmentation, drug susceptibility, catalase activity, colony aspect, cell habitus, etc.) the name *Mycobacterium luciflavum* may perhaps be more justified.

The exact role played by this organism in producing pulmonary disease is not quite clear yet. The regular and repeated recovery of large numbers of this bacterium from the sputum together with the clinical and roentgenografic findings of the three patients concerned are highly suggestive for establishing *Myc. luciflavum* as the etiologic agent of a rare form of tuberculosis. This conception is strengthened by several records on the isolation of this species, or sometimes related chromogenic forms, from resected pulmonary lesions strongly resembling normal tuberculosis (6, 8, 22–24). Consequently, as appears from the literature, most investigators tend to consider these species as the direct cause of certain human infections, be it that some authors also take into account the possibility that these organisms may eventually have become established in old tuberculous lesions as a result of secundary invasion (6, 23).

As previously mentioned the photochromogenic strains investigated displayed some degree of natural resistance to some of the more potent antitubercular agents; notably to I.N.H. and to p-aminosalicylic acid these organisms appeared to be about 20 times more resistant than *Myc. tuberculosis*. In accord with these in vitro observations the results of chemotherapy of infections due to these organisms appear from some records (6, 23, 25) to be rather disappointing. It follows that involvement of an atypical *Mycobacterium* in pulmonary disease is a condition which should not be disregarded.

## Summary.

Three strains of atypical photochromogenic mycobacteria are described which have repeatedly been isolated from the sputum of three patients with pulmonary disease highly simulating tuberculosis.

All three strains were identical on account of the following properties: production of a light-conditioned lemon-yellow pigment, texture and aspect of colonies, poor tendency of cord formation, negative neutral red reaction, stout cell habitus, and low degree of pathogenicity to the guinea pig.

The susceptibility of the strains studied to isonicotinic acid hydrazide, p-aminosalicylic acid, thiosemicarbazone, streptomycin, viomycin, cycloserin, and tetracyclin exhibited a striking similarity

with the sensitivity pattern of *Myc. avium*. Unlike the latter organism, the photochromogenic organisms displayed the strong catalase activity characteristic of saprophytic mycobacteria, whilst this activity was fully maintained in the mutants being resistant to 10 μg/ml of I.N.H.

Some of the properties which distinguishes the photochromogenic mycobacteria from *Myc. tuberculosis* are shortly discussed.

As a name for this organism is proposed *Mycobacterium luciflavum*.

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# A NATURALLY OCCURRING HIGHLY ATTENUATED STRAIN OF TYPE 1 POLIOMYELITIS VIRUS

by

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The incidence of poliomyelitis in the temperate areas of the northern hemisphere is usually lowest in winter and spring. The months of January through June 1956 were just in between the seasonal peaks of 1955 and 1956 in the Netherlands. The cases of poliomyelitis reported in January were apparently the last ones of the 1955 season, and the cases reported in June were representatives of the beginning 1956 outbreak. From February through May, only a few isolated cases have been reported. In order to look for the occurrence of natural strains of poliomyelitis virus showing a low grade of neurotropism, 20 strains isolated between January and June were examined for neurotropism.

Since Sabin stated that neurotropism is not an all or none character, but that different strains exhibit spectrums of activity from high to low, only strains isolated from cases of minor illness and from healthy contacts were selected for this purpose. According to the fact that Sabin found the index of neurotropism for primates to be not only a function of the virus but also of the neurons, the strains were tested primarily by intracerebral inoculation of *Cynomolgus* monkeys. Those, who failed to produce paralysis following this mode of infection, were inoculated intraspinally.

Of all strains tested, 18 type 1 strains and 1 type 2 strain produced paralysis following intracerebral inoculation of 0.5 ml of undiluted tissue culture fluid. One type 1 strain, which will be referred to as

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[1]) Aided with a grant from the National Health Research Council T.N.O.

strain Leiden 1956/32, however, appeared to deserve further attention.

This strain has been isolated from a woman, 37 years of age, who was admitted to the hospital on January 7th, 1956, because of acute abdominal pain, nausea and vomiting. Appendectomy was performed, but the appendix showed no lesions. Since BORNHOLM'S disease could not be excluded, a stool specimen collected on January 14th was examined virologically by inoculation of suckling-mice and monkey kidney culture. The mice did not show any signs of illness, but in tissue culture a transmissible cytopathogenic agent was recovered. Neutralization tests with type-specific poliomyelitis monkey immune serums revealed the presence of type 1 poliomyelitis virus. Moreover, suckling-mice were inoculated with undiluted tissue culture fluid, but none developed any signs of illness, so that Coxsackie virus could be excluded.

No virus was isolated from stools collected on February 12th and 27th.

The second tissue culture passage of the type 1 strain isolated from the first stool specimen proved non-paralytogenic for 2 intracerebrally inoculated *Cynomolgus* monkeys, since no paralysis developed following inoculation of 0.5 ml representing approximately 5 million $TCD_{50}$. Although no histological lesions were found in the spinal cord of one monkey that had been sacrificed 9 days after inoculation, the virus could be recovered from the cervical enlargement. This monkey passage virus, like the original tissue culture virus, failed to produce paralysis and cord lesions in 2 *Cynomolgus* monkeys following intracerebral inoculation of a 10 per cent cord suspension.

The virus was again isolated from the original stool specimen, which had been stored in the frozen state. Undiluted fluid from the first tissue culture passage of the second virus yield was inoculated into 2 *Cynomolgus* monkeys, each of them receiving 0.5 ml in both hemispheres, 0.1 ml intraspinally and 1 ml intramuscularly. No paralysis developed and no cord lesions were found in the animals when sacrificed 7 and 14 days after inoculation.

Thus, there was circumstantial evidence that the stools of the patient involved contained a naturally occurring type 1 poliomyelitis virus of very low neurotropism. In order to ascertain whether this viral population consisted of particles possessing a uniformly low neurotropism, or whether it might consist of particles with varying neurotropism, 6 single particle cultures were prepared from a limiting dilution of the 5th tissue culture passage by the plaque method in monolayer cultures of trypsinized monkey kidney cells (Dulbecco and Vogt, 1954). The progeny of each of the plaques was inoculated into 2 *Cynomolgus* monkeys: 0.5 ml in both of the hemispheres, 0.1 ml intraspinally and 1 ml intramuscularly, all quantities as undiluted tissue culture fluid (titre $10^{7.0}$/ml). The progeny of two plaques which failed to produce paralysis was triply purified, and then inoculated intraspinally as undiluted tissue culture fluid and in the dilutions $10^{-1}$ and $10^{-2}$, into 5 *Cynomolgus* monkeys each. The results of this test are summarized in table 1.

TABLE 1.

Results of intraspinal inoculation of progeny of 6 different plaques of strain Leiden 1956/32.

| Plaque progeny | Number of monkeys: paralysis/inoculated | | | Cord lesions |
|---|---|---|---|---|
| | undiluted | $10^{-1}$ | $10^{-2}$ | |
| A | 2/2 | | | + |
| B | 0/2 | | | — |
| C | 0/2 | | | + |
| D | 0/2 | | | — |
| E | 0/2 | | | + |
| F | 0/2 | | | — |
| E (triply purified) | 3/5 | | | + |
| F (triply purified) | | 0/5 | 0/5 | + |

Table 1 shows, that the type 1 strain involved is actually a mixture of virus particles with varying degree of neurotropism, the majority of them, however, possessing a low neurotropic activity. This is probably the reason why undiluted tissue culture fluid of the crude virus appeared to be highly attenuated. The non-paralytogenic properties of this material might be due to an interfering effect between the non-paralytogenic and the paralytogenic virus particles.

On our request, Dr A. B. Sabin (Children's Hospital Research Foundation, Cincinnati, Ohio, U.S.A.) was kind enough to examine in his laboratory the same strain. The results of this examination are summarized in table 2.

TABLE 2.

Spinal activity in monkeys of triply purified plaque progeny derived from strain Leiden 1956/32 (Dr A. B. SABIN).

| Plaque progeny | Number of monkeys: paralysis/inoculated | | |
|---|---|---|---|
| | undiluted | $10^{-1}$ | $10^{-2}$ |
| original fluid | 3 + 2?/10 | 1?/5 | 2/5 |
| plaque 1 | 1/4 | | 1/5 |
| ,, 2 | 1?/5 | | 1/5 |
| ,, 3 | 2/5 | | 1/5 |
| ,, 4 | 1/5 | | 1/5 |
| ,, 5 | 1?/5 | | 0/5 |
| | 3/5 | | 0/5 |
| | 2/10 | 3/5 | 1/5 |

It appears from these results also, that the Leiden 1956/32 strain behaved like a highly attenuated virus, but it exhibited the tendency for "zoning", since the original fluid produced paralysis in a lower percentage of monkeys inoculated intraspinally with the $10^{-1}$ than with the $10^{-2}$ dilution. Likewise, the progeny of plaque 5, in the third series of monkeys, produced paralysis in a lower percentage following inoculation of undiluted fluid than following inoculation of the $10^{-1}$ dilution. This phenomenon seems to be a property of the individual virus particles rather than the result of a mixture of virus particles possessing different properties. When testing 10 individual plaques from the LSc strain, SABIN observed this phenomenon in the progeny of 9 plaques. Only one did not exhibit the zoning phenomenon, and that is why it has been chosen for immunization of human volunteers.

Comparison of tables 1 and 2 also shows, that testing single particle cultures for neutrotropic activity requires at least 5 monkeys per dilution in order to be able to draw conclusions.

The immunizing capacity of strain Leiden 1956/32 has been examined by inoculation of 2 groups of 6 *Cynomolgus* monkeys, one group receiving 0.3 ml of undiluted tissue culture fluid intracutaneously, and the other 0.5 ml subcutaneously. Two months later, both groups and a control group of 6 monkeys were fed 5 million $TCD_{50}$ of the Mahoney strain mixed with cream milk. None of the immunized monkeys and 4 monkeys of the control group (67%) developed paralysis.

The antigenic capacity of the strain has been determined by 3 subcutaneous injections of 2 ml of undiluted culture fluid into 10 guinea pigs with one week interval, and by 3 subcutaneous injections of 2 ml of undiluted culture fluid inactivated by formalin 1/4000 into another group of 10 guinea pigs. By using the pH test, no antibody was present in the pre-vaccination serums of both groups. In pooled serum samples collected 14 days after the last injection, the type 1 antibody titre in the group inoculated with live virus was 1/64 (original serum dilution), and in the group inoculated with formalin-inactivated virus 1/32. Moreover, in the post-vaccination serum pools, a low level of heterotypic antibody (1/4 against type 2 and type 3) was found in both groups.

## Summary.

Twenty strains of poliomyelitis virus recovered from cases of abortive, non-paralytic, poliomyelitis have been tested for neurotropism. Only one strain possessed a low intraspinal activity in *Cynomolgus* monkeys. Six single particle cultures were derived from this naturally occurring highly attenuated strain of type 1 poliomyelitis virus. It has been shown that the original virus consisted of a mixture of virus particles with varying degree of neurotropism. The antigenic and immunizing capacity of one highly attenuated plaque progeny has been tested in guinea pigs and monkeys.

## Acknowledgement.

We are much indebted to Dr A. B. Sabin for his kindness to test the strain in a large number of monkeys.

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# ON METHANE FERMENTATION OF HIGHER ALKANES

by

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## § 1. INTRODUCTION.

The literature on the attack of hydrocarbons and petroleum by aerobic bacteria is fairly extensive (see (10)), but data on the attack by anaerobic organisms are scarce. To the writer's knowledge the only ones are papers by TAUSSON and ALESHINA (6) and by TAUSSON and VESELOV (7) and a couple of short notes by NOVELLI and ZOBELL (4) and by ZOBELL's coworker ROSENFELD (5) on the action of sulphate-reducing bacteria [1]).

TAUSSON and ALESHINA brought glass powder coated with paraffin wax and bees wax resp. in a mineral medium inoculated with sulphate-reducing bacteria and observed a blackening of the coated glass particles after a few days (the blackening indicating $H_2S$ production at the particle surface). These cultures could be transferred on the same medium containing glass powder coated with paraffin wax and bees wax. Similar results were obtained with a Baku crude oil dispersed in a pasty mixture of calcium sulphate, calcium carbonate and kaolin.

TAUSSON and VESELOV conducted similar experiments with phenanthrene and naphthalene and obtained a distinct growth of the bacteria, coupled with an erosion of the crystals of these aromatic hydrocarbons.

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[1]) It is true that THAYSEN (8) observed methane production in the water bottom of a kerosene tank in which an explosion had occurred and reports the isolation of a bacterium which fermented kerosine to methane. However, the statement that besides methane, also acetaldehyde, lactic and acetic acids were produced and that also nitrate should be present, make it seem doubtful whether a real methane fermentation of alkanes occurred.

NOVELLI and ZOBELL obtained growth of sulphate-reducing bacteria on a mineral medium containing higher alkanes. Growth on decane was weak, somewhat better on tetradecane and quite evident on eicosane and higher alkanes including paraffin oil and paraffin wax, which showed a marked attack. Neither aromatics nor lower alkanes yielded growth.

Multiplication of the bacteria and attack of the hydrocarbons were accompanied by production of hydrogen sulphide. The hydrocarbons were adsorbed on sand particles. Concerning the fate of the substrates it is stated that they are partly oxidized, partly assimilated and partly converted to lower alkanes.

ROSENFELD employed a somewhat different technique in that he emulsified the substrates in gum arabic (which by itself was not attacked).

He obtained sulphate reduction with Californian and Pennsylvanian crude oils, kerosine, lubricating oil and paraffin oil and reports the occurrence of insoluble fatty acids as intermediates. ROSENFELD further established the conversion of hexadecene and observed reduction in Thunberg tubes of methylene blue in the presence of hexadecane and hexadecene by washed cells from a pure culture. Other anaerobic bacteria failed to reduce methylene blue under these conditions; no reduction occurred with gum arabic instead of the hydrocarbons.

It should be noted that UPDEGRAFF and WREN (9) could not confirm the results of NOVELLI and ZOBELL and of ROSENFELD.

Complete dehydrogenation of higher alkanes with sulphate as the hydrogen acceptor can be formulated as follows:

$$4\,(CH_2) + 3\,SO_4'' = 4\,HCO_3' + 2\,SH' + H_2S.$$

The work of BARKER (2) showed that there exists a close analog between sulphate reduction and methane fermentation of substrates like alkanols and lower fatty acids (with the exception of acetic acid), the latter process consisting of a dehydrogenation of these substrates with carbon dioxide as a hydrogen acceptor.This analogy induced Prof. Dr A. J. KLUYVER and Dr G. Th. PHILIPPI, as well as the writer in 1948 to postulate an analogous methane fermentation of higher alkanes according to the formula:

$$4\,(CH_2) + 3\,CO_2 + 2\,H_2O = 4\,CO_2 + 3\,CH_4 \text{ or}$$
$$4\,(CH_2) + 2\,H_2O = CO_2 + 3\,CH_4.$$

It was then decided to try to verify this hypothesis experimentally which work was carried out by the writer in the years 1948–1953.

Mention should be made here of an investigation by KUZNETZOV (3) who postulated a microbial production of methane from crude oils. He could isolate methane bacteria from samples of crude oil and of water and cores from rocks of oil-bearing formations, but failed to demonstrate methane production from heptane. With a view to the results of NOVELLI and ZOBELL and of ROSENFELD he might have been more successful if he had used higher alkanes instead of heptane.

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## § 2. MATERIALS AND METHODS EMPLOYED.

The following substrates were taken as sources of higher alkanes:

Indonesian light paraffinous crude oil (L P O I),
Indonesian heavy paraffinous crude oil (H P O I),
Indonesian heavy asphaltic crude oil (H A O I),
Indonesian extra heavy paraffinous crude oil (E H P O I),
Netherlands heavy paraffinous crude oil (H P O N),
Venezuelan light paraffinous crude oil (L P O V),
Venezuelan medium mixed type crude oil (M M O V),
Venezuelan heavy mixed type crude oil (H M O V),
Mid-Eastern heavy paraffinous crude oil (H P O E),
Mid-Eastern light paraffinous crude oil (L P O E 1),
Mid-Eastern light paraffinous crude oil (L P O E 2),
Crude paraffin wax (C P W),
Isoalkanes isolated from same (I C P W),
Moderately purified paraffin wax (M P W),
Pure paraffin wax (P P W),
Medicinal paraffin oil (P O),
Pure hexadecane (H D).

The inoculum was obtained from six sources, *viz.*, black mud from tidal flats, horse-dung, sludge from the waste liquor settling pond of a strawboard mill, crude oil from well-heads of a Netherlands oil field and cultures of methane bacteria on calcium acetate or ethanol which were transferred from enrichment cultures started with strawboard waste sludge.

Since bacteria can only develop in an aqueous medium, it is desirable to make the contact area between the (insoluble) substrate and the water as large as possible. In the present work this aim was usually achieved by stirring the oils or the molten waxes into a paste of clay [1]) and water [2]) containing the following mineral nutrient salts: $NH_4Cl$ 0.10%, $K_2HPO_4$ 0.05%, $MgSO_4 . 7\,aq$ 0.01%.

In order to remove the last traces of oxygen the medium was boiled shortly before use and one drop of a 10% solution of $Na_2S.9\,aq$ added per 100 ml of medium.

In some cases asbestos fibres coated with oil or paraffin wax were used instead of a dispersion in clay paste.

For the enrichment cultures and their subcultures 1% Ca-acetate or ethanol was added to this medium; the subcultures contained a 4 cm layer of asbestos fibres.

The inoculum was added to the clay paste before the oil or wax was stirred in.

Successful enrichment cultures on Ca-acetate were obtained at 30, 45 and 55°C. Cultures in 250 ml bottles reached peak gas productions of 180, 240 and 450 ml/day. Apparently the methane bacteria in the strawboard waste sludge are able to adapt themselves to a wide range of temperatures [3]).

Most cultures were started in 1 l bottles containing about 570 g of air-dry clay and about 50 g of oil or wax. The inoculum was either 100 g of mud, sludge, horse-dung and crude oil or the entire contents of a 250 ml enrichment culture.

Some cultures were started in 10 l bottles containing tenfold amounts of the respective constituents. The bottles were filled up with the aqueous medium. The gas produced by the cultures was collected over saturated NaCl solution in inverted calibrated cylinders standing in beakers. All gas produced was analyzed for $CO_2$ by absorption in alkali and for $CH_4$ by combustion over copper oxide, followed by absorption of the $CO_2$ formed.

Most cultures were incubated at 30°C. and some at 55°C. As strawboard waste sludge and horse-dung yielded appreciable quantities of gas by itself [1]), a culture containing sludge, or dung resp., but no oil or wax ("sludge blank") was added to each series of cultures started simultaneously. Moreover, in practically all series cultures were included containing clay and oil or wax but no inoculum ("oil blanks").

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[1]) The best results were obtained with a heavy young sea silt with about 16% clay ($< 16\mu$).

[2]) Water from Indonesian oil wells containing about 0.8% $HCO_3'$ 0.3% $Cl'$, 0.5% $Na^{\cdot} + K^{\cdot}$ and small amounts of $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$ and $SO_4''$ gave the best results.

[3]) To the writer's knowledge this is the first report of a methane fermentation occurring a 45°C.

When a culture was broken off the entire contents were transferred to separation funnels and washed with pentane until the aqueous layer did not contain any visible amounts of oil. The pentane solution was dried over anhydrous sodium sulphate and the pentane distilled off. The oil or wax recovered from some of the cultures was analyzed at the Royal Dutch-Shell Laboratory at Amsterdam (see § 4).

## § 3. GAS PRODUCTION FROM CULTURES CONTAINING OIL OR WAX.

Total gas, methane and carbon dioxide production of nearly all cultures are listed in table 1 [2]).

Not listed are the cultures at 55°C. and those inoculated with mud from tidal flats and with cultures of methane bacteria on ethanol, as well as the cultures in 10 l bottles.

The former yielded little or no gas, while the latter gave results similar to, although less favourable than corresponding 1 l cultures.

Also omitted from table 1 are the oil blanks of $\varphi$ a and $\varphi$ c. These blanks were added with the intention to have a check on the recovery procedure of the oil from corresponding cultures inoculated with strawboard waste sludge. In order to prevent any development of methane bacteria in these bottles 5 ml 36% formaldehyde solution were added to each 1 l bottle. When after 42 days nevertheless some gas production occurred in these blanks another 5 ml 36% solution were added. However gas production continued, with total methane productions of 1390 and 1410 ml of $CH_4$ in the course of $2\frac{1}{2}$ years. Since the $CH_4/CO_2$ ratio of the gas produced was always much lower than in the gas from successful inoculated cultures (see below), it was evident that a different process had occurred in these oil blanks.

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[1]) This sludge always contains a certain amount of carbohydrate-like matter which is susceptible to methane fermentation.

[2]) The sum of $CH_4$ and $CO_2$ is always somewhat lower than total gas. As $O_2$, when looked for, was always found to be absent, the remainder must be nitrogen originating from air getting into the gas collecting system, the $O_2$ being consumed by aerobic bacteria which are always potentially present.

TABLE 1.

Total gas, methane and carbon dioxide production of cultures at 30°C.

| Culture | Age months | Composition | Gas ml | $CH_4$ ml | $CO_2$ ml |
|---|---|---|---|---|---|
| ηlc | 56 | HPOI, acetate culture | 6.280 | 4.610 | 1.210 |
| φa | 35 | HAOI, strawboard waste sludge | 800 | 400 | — |
| φb | 55 | LPOI, strawboard waste sludge | 490 | 290 | 80 |
| φc | 55 | HPOI, strawboard waste sludge | 8.290 | 6.120 | 1.790 |
| φe | 55 | Sludge only | 1.550 | 830 | 490 |
| ψa | 51 | HAOI, horse-dung | 7.840 | 4.530 | — |
| ψa bl | 51 | HAOI only | 400 | 200 | — |
| ψb | 51 | LPOI, horse-dung | 4.700 | 2.750 | — |
| ψb bl | — | LPOI only | 0 | — | — |
| ψc | 51 | HPOI, horse-dung | 8.780 | 4.950 | — |
| ψc bl | 51 | HPOI only | 440 | 190 | — |
| ψd | 18 | horse-dung only | 9.470 | 4.910 | — |
| ψe | 51 | horse-dung only | 9.080 | 5.020 | — |
| ωa | 36 | HAOI only | 360 | 130 | — |
| ωb | — | LPQI only | 0 | — | — |
| ωc | 54 | ZPOI only | 380 | 210 | — |
| C 1 | 44 | paraffine oil, strawboard waste sludge | 1.660 | 900 | 540 |
| C 2 | 44 | paraffine oil only | 580 | 330 | 130 |
| C 3 | 48 | HPOI, strawboard waste sludge | 6.290 | 4.590 | 1.250 |
| C 4 | 48 | HPOI only | 380 | 190 | 60 |
| C 5 | 48 | sludge only | 1.470 | 860 | 460 |
| D 1 | 46 | Topped LPOI, strawboard waste sludge | 4.330 | 3.040 | 990 |
| D 2 | 46 | Topped LOPI only | 180 | 80 | — |
| D 3 | 45 | LPOI, strawboard waste sludge | 560 | 310 | 120 |
| D 4 | 18 | LPOI only | 20 | — | — |
| D 5 | 46 | HPOI, strawboard waste sludge | 6.220 | 4.330 | 1.380 |
| D 6 | 46 | HPOI only | 280 | 170 | 50 |
| D 7 | 46 | Sludge only | 1.720 | 970 | 600 |
| F 2 | 6 | HPOI, strawboard waste sludge | 1.640 | 910 | 600 |
| F 5 | 12 | Idem | 5.750 | 3.910 | 1.490 |
| F 3 | 24 | Idem | 6.800 | 4.770 | 1.570 |
| F 6 | 30 | Idem | 7.660 | 5.460 | 1.780 |
| F 4 | 36 | Idem | 7.890 | 5.600 | 1.830 |
| F 1 | 44 | Idem | 9.180 | 6.490 | 1.990 |
| F 7 | 44 | Sludge only | 1.540 | 770 | 500 |
| G 1 | 36 | HPON, strawboard waste sludge | 1.560 | 970 | 460 |
| G 2 | 36 | HPON only | 130 | 60 | — |
| G 3 | 28 | LPOV, strawboard waste sludge | 480 | 260 | — |
| G 4 | 28 | LPOV only | 0 | — | — |
| G 5 | 36 | MMOV, strawboard waste sludge | 710 | 420 | 170 |
| G 5′ | 36 | Idem | 690 | 370 | 190 |

TABLE I (continued).

| Culture | Age months | Composition | gas ml | $CH_4$ ml | $CO^2$ ml |
|---|---|---|---|---|---|
| G 6 | 36 | M M O V only | 0 | —— | —— |
| G 7 | 17 | H M O V, strawboard waste sludge | 230 | 110 | 50 |
| G 8 | 17 | H M O V only | 0 | —— | —— |
| G 9 | 37 | H P O I, strawboard waste sludge | 6.200 | 4.450 | 1.280 |
| G 10 | 14 | Sludge only | 580 | 260 | 230 |
| H 1–14 | 18 | H P O N or H P O I, crude from well-heads | 100 | —— | —— |
| J 1 | 9 | L P O E 1, strawboard waste sludge | 240 | 60 | 130 |
| J 2 | 9 | L P O E 1 only | 40 | —— | —— |
| J 3 | 31 | H P O E, strawboard waste sludge | 4.100 | 2.800 | 1.040 |
| J 4 | 31 | H P O E only | 210 | 130 | 50 |
| J 5 | 16 | L P O E 2, strawboard waste sludge | 440 | 200 | —— |
| J 6 | 16 | L P O E 2 only | 20 | —— | —— |
| J 7 | 31 | C P W, strawboard waste sludge | 21.240 | 15.920 | 4.210 |
| J 8 | 31 | C P W only | 13.440 | 10.000 | 2.650 |
| J 9 | 31 | I C P W, strawboard waste sludge | 11.580 | 8.550 | 2.460 |
| J 10 | 31 | I C P W only | 1.230 | 880 | 240 |
| J 11 | 29 | M P W, strawboard waste sludge | 26.040 | 19.740 | 5.030 |
| J 12 | 31 | M P W only | 220 | 130 | —— |
| J 13 | 22 | P P W, strawboard waste sludge | 22.510 | 16.920 | 4.380 |
| J 14 | 30 | P P W only | 300 | 180 | 40 |
| J 15 | 29 | H D, strawboard waste sludge | 1.580 | 980 | 470 |
| J 16 | 29 | H D only | 470 | 260 | 140 |
| J 17 | 30 | H P O I, strawboard waste sludge | 7.090 | 5.300 | 1.400 |
| J 18 | 31 | Sludge only | 1.190 | 710 | 330 |
| K 1 | 26 | H P O N, strawboard waste sludge | 3.920 | 2.720 | 930 |
| K 2 | 26 | H P O N only | 270 | 170 | —— |
| K 3 | 25 | L P O V, strawboard waste sludge | 520 | 240 | 190 |
| K 4 | 25 | L P O V only | 20 | —— | —— |
| K 5 | 25 | M M O V, strawboard waste sludge | 880 | 480 | 280 |
| K 6 | 25 | M M O V only | 10 | —— | —— |
| K 7 | 22 | H M O V, strawboard waste sludge | 410 | 190 | 150 |
| K 8 | 22 | H M O V only | 20 | —— | —— |
| K 9 | 27 | E H P O I, strawboard waste sludge | 12.230 | 9.250 | 2.330 |
| K 10 | 27 | E H P O I only | 10 | —— | —— |
| K 14 | 27 | P P W, strawboard waste sludge | 26.170 | 20.010 | 4.890 |
| K 15 | 27 | Sludge only | 1.190 | 730 | 320 |
| L 1 | 21 | H P O I, clay, strawboard waste sludge | 7.330 | 5.420 | —— |
| L 2 | 21 | H P O I, asbestos, strawboard waste sludge | 2.840 | 2.230 | —— |
| L 5 | 21 | Clay and sludge only | 1.800 | 1.180 | —— |
| L 6 | 21 | Asbestos and sludge only | 1.030 | 780 | —— |

This led to the hypothesis that a methane fermentation of formaldehyde had occurred in the bottles, according to the equation:

$$2\,CH_2O = CH_4 + CO_2$$

A separate investigation (1) showed that a culture of methane bacteria on Ca-acetate is indeed able to ferment formaldehyde to methane and carbon dioxide. As no inoculum was added to the oil blanks, the methane bacteria must either have got in as a contamination or have originated from the clay added to these bottles.

The cultures in table 1 are arranged in series ($\varphi$, $\psi$, C, D, etc.) which were started simultaneously. The cultures containing Indonesian heavy asphaltic and light paraffinous oils (HAOI and LPOI), the Venezuelan oils (LPOV, MMOV and HMOV), the Mid-Eastern light paraffinous oils (LPOE 1 and 2), paraffin oil and hexadecane yielded only limited amounts of gas which did not surpass the gas production of the sludge blanks of the respective series[1]). These negative results are not due to weakness of the inoculum, as the respective series always contained successful oil or wax cultures.

Negative results were also obtained with the cultures inoculated with horse-dung (series $\psi$) or crude from well-heads. Apparently the horse-dung employed, although yielding a vigorous methane fermentation of its own (see $\psi$ d and e), did not contain any bacteria capable of attacking hydrocarbons.

The other inoculated cultures listed in table 1 all yielded appreciably higher gas volumes than their respective sludge blanks.

This becomes evident from table 2 which shows the net methane production of the successful cultures, i.e. the difference between the production of the culture and that of the corresponding sludge blank.

Moreover, table 2 contains data on the $CH_4/CO_2$ ratio of the total gas production and on the hydrocarbon equivalent of the net $CH_4$ production calculated on the basis of the equation for complete conversion of higher alkanes:

$$4\,(CH_2) + 2\,H_2O = 3\,CH_4 + CO_2$$

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[1]) The hexadecane culture J 15 shows a surplus of 270 ml $CH_4$ over the sludge blank J 18. Taking into account the age of these cultures (about 30 months), this surplus is considered insignificant.

TABLE 2.

Methane production of successful cultures.

| Culture | Oil or wax | Age months | Net $CH_4$ prod., ml. | $CH_4/CO^2$ ratio | Hydrocarbon equid., g |
|---|---|---|---|---|---|
| F 2 | H P O I | 6 | 340 | 1.5 | 0.26 |
| F 5 | ,, | 12 | 3.420 | 2.6 | 2.56 |
| L 1 | ,, | 21 | 4.240 | — | — |
| L 2 | ,, | 21 | 1.450 | — | — |
| F 3 | ,, | 24 | 4.080 | 3.0 | 3.06 |
| F 6 | ,, | 30 | 4.700 | 3.1 | 3.53 |
| J 17 | ,, | 30 | 4.610 | 3.8 | 3.46 |
| F 4 | ,, | 36 | 4.790 | 3.1 | 3.59 |
| G 9 | ,, | 37 | 4.190 | 3.5 | 3.14 |
| F 1 | ,, | 44 | 5.720 | 3.3 | 4.30 |
| D 5 | ,, | 46 | 3.360 | 3.1 | 2.52 |
| C 3 | ,, | 48 | 3.730 | 3.7 | 2.80 |
| φc | ,, | 55 | 5.290 | 3.4 | 3.97 |
| ηlc | ,, | 56 | 3.980 | 3.8 | 2.99 |
| D 1 | Topped L P O I | 46 | 2.070 | 3.1 | 1.55 |
| K 9 | E H P O I | 27 | 8.520 | 4.0 | 6.40 |
| K 1 | H P O N | 26 | 1.990 | 2.9 | 1.49 |
| G 1 | ,, | 36 | 710 | 2.1 | 0.53 |
| J 3 | H P O E | 31 | 2.090 | 2.7 | 1.57 |
| J 7 | C P W | 31 | 14.890 | 3.8 | 11.18 |
| J 8 | ,, (no inoculum) | 31 | 9.890 | 4.5 | 7.41 |
| J 9 | I C P W | 31 | 6.660 | 3.5 | 5.00 |
| J 10 | ,, (no inoculum) | 31 | 880 | 3.7 | 0.66 |
| J 11 | M P W | 29 | 19.050 | 3.9 | 14.30 |
| J 13 | P P W | 22 | 16.290 | 3.9 | 12.22 |
| K 14 | P P W | 27 | 18.980 | 4.1 | 14.22 |

according to the formula:

$$\frac{\text{net CH}_4 \text{ in l}}{22.4} \times \frac{273}{303} \times \frac{4}{3} \times 14 \text{ g.}$$

The Indonesian heavy paraffinous oil (H P O I) was used as a standard material checking the viability of the inoculum and always yielded a fair amount of gas. In the cultures of the F series (which contained exactly the same inoculum) there is a regular increase of methane production with the age at which the culture was broken off.

The other H P O I cultures, belonging to different series show

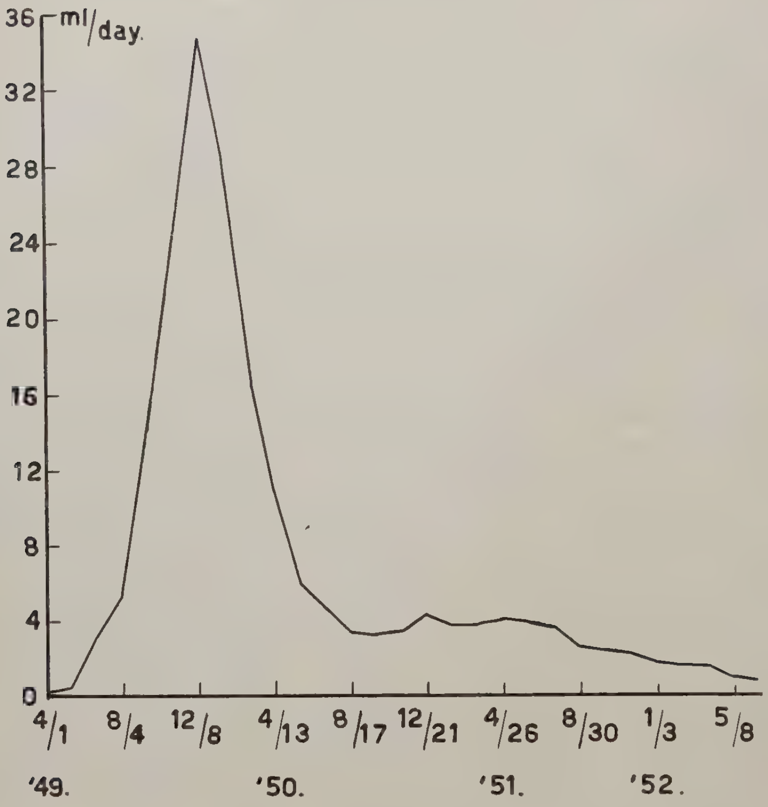

Fig. 1. Mean daily gas production during six-weekly periods of culture $\varphi$C.

proportionally smaller yields, probably because of some variation in the amount of oil in the culture and of a lower activity of the bacteria in the inoculum 1).

The course of gas production in these cultures shows a comparatively steep rise to a maximum (varying between 12 ml/day for D 5 and 36 ml/day for $\varphi$c), followed by a gradual drop to a level of 3–4 ml/day which was maintained for a year or more and then dropped very slowly to zero. As an illustration the total gas production curve from the 4th to the 38th month of culture $\varphi$ c is shown in fig. 1.

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1) Culture $\eta 1$ c was inoculated with an acetate culture derived from strawboard waste sludge, the others directly with sludge. In calculating the net production of $\eta 1$ c allowance was made for the acetate introduced with the inoculum. The activity of the bacteria in the sludge probably varied in the course of time.

Comparison of the figures for L 1 and L 2 in table 2 shows that dispersing the oil in a clay paste is much more effective than absorbing it in asbestos fibres.

Gas production with the other oils and with the waxes followed a similar course, with one notable exception, *viz.*, D 1. The failure of gas production in cultures with the Indonesian light paraffinous oil (LPOI) $\eta$l b (not listed in table 1) and $\varphi$ b (although this crude contains more higher alkanes than the HPOI) led to the hypothesis that development of methane bacteria in these cultures was hampered by the high aromatic content in the gasoline fraction of this crude (e.g. benzene and toluene which are quite toxic to bacteria in general).

Therefore, a culture was started with LPOI from which the fraction up to a boiling point of 200°C had been distilled off (topped LPOI). Gas production in this culture started very slowly, reached a rate of about 4 ml/day at the age of 21 months and continued at this rate until it was broken off at the age of 46 months. The net $CH_4$ production of this culture shows that removal of the gasoline fraction indeed enabled the methane bacteria to develop, although this development was still much slower than that of the HPOI cultures. The failure to produce gas from the LPOV may be due to the same cause as in the case of the LPOI. A few analytical data may elucidate this point:

| Oil | Fraction 180° | | Fr. 180–250° | | Fr. 250–325° | | Fr. 325–400° | | Fr. 400 |
|---|---|---|---|---|---|---|---|---|---|
| | % wt. on total crude | % wt. of aromatics on fraction | % wt. on total crude | % CA 1) | % wt. on total crude | % wt. of cryst. on fraction | % wt. on total crude | % wt. of cryst. on fraction | % wt. on total crude |
| HAOI | 0.0 | — | 11.0 | 5 | 27.6 | 0 | 26.6 | 0 | 34.8 |
| HPOI | 6.7 | 9 | 12.3 | 15 | 29.5 | 3.0 | 17.6 | 15.9 | 33.9 |
| LPOI | 23.0 | 50 | 22.5 | 30 | 15.2 | 15.1 | 14.8 | 29.1 | 24.5 |
| LPOV | 21.8 | 12 | 14.6 | 8 | 15.4 | 12.0 | 14.8 | 12.9 | 33.4 |
| MMOV | 11.3 | 13 | 10.0 | 12 | 10.9 | 0.0 | 15.3 | 0.0 | 52.5 |
| HMOV | 8.7 | 11 | 12.6 | 15 | 12.9 | 0.0 | 16.0 | 0.0 | 49.8 |

1) % CA = % of total carbon in aromatic rings.

Thus, it appears that the LPOI contains 11.5% low-boiling aromatics, against 0.6% for the HPOI.

For the LPOV this figure is 2.6% which is much lower than for the LPOI, but still appreciably higher than for the ZPOI. The HAOI has no low-boiling fraction, while in the MMOV and HMOV the % of low-boiling aromatics on total crude is also comparatively low (1.5 and 1.0% resp.). However, these oils do not contain any crystallizable paraffin wax in their higher-boiling fractions, so that they are lacking in higher alkanes. This point will be dealt with in more detail in § 5.

The Indonesian extra-heavy paraffinous oil (EHPOI) showed a much higher net production which is in line with its much higher wax content (see § 4). Comparatively small productions occurred in the cultures with the Netherlands and Mid-Eastern heavy paraffinous oils (HPON and HPOI; the difference between the HPON cultures G 1 and K 1 is probably due to a difference in the inoculum). The wax content of the HPOE is much smaller than that of the HPOI, but that of the HPON is of the same order of magnitude.

Still larger volumes of gas were produced by the inoculated cultures with waxes, with the exception of the iso-alkanes from the crude paraffin wax (ICPW) which are apparently less susceptible to attack by methane bacteria than the normal alkanes. The course of the gas production is similar to that of the HPOI cultures, but the maxima are much higher (up to 134 ml/day in K 14).

The same holds for the level to which gas production drops after the maximum (e.g. in J 13 to 24, later on rising again to 32ml/day).

In most cultures showing an appreciable gas production the $CH_4/CO_2$ ratio lies between 3.0 and 4.0, i.e. close to the theoretical one of 3.0 (on the basis of the equation given above).

Lower values are explained by the influence of the fermentation of sludge components (the sludge blanks showing ratios between 1.1 and 2.3) which influence will be stronger the lower the total gas production of the cultures (see e.g. F 2 and G 1).

The figures for the theoretical hydrocarbon equivalent of the net methane production show considerable conversions in the case of the wax cultures J 7, J 11, J 13 and K 14.

The gas production in the not-inoculated "oil blanks" J 8 and J 10 constitutes a remarkable phenomenon, showing that the presence of a lot of complex organic matter in the form of strawboard waste sludge is not an essential condition for methane production from the waxes involved. The very small amounts of methane evolved in the other oil blanks in table 1 already show that in these blanks methane bacteria do occur (originating either from contamination or from the clay) which, however, are usually unable to adapt themselves to a fermentation of higher alkanes.

In one case (culture F 2) the contents of the bottle were examined microscopically. Many small rod-shaped bacteria were noticed among the oil droplets.

## § 4. RECOVERY AND ANALYSIS OF RESIDUAL OIL AND WAX FROM CULTURES.

Preliminary experiments on the recovery of the H A O I, H P O I and L P O I from freshly prepared clay-oil-water mixtures showed that the former two crudes could be recovered with little or no loss of oil, but that with the L P O I a loss of about 13% occurred. Cultures which contained strawboard waste sludge besides clay, oil and water usually showed greater losses, even in cases where apparently no methane fermentation of hydrocarbons had developed.

In cultures with a well-developed fermentation the loss was sometimes excessive (e.g. 52% in F 1) and much higher than the hydrocarbon equivalent of the net $CH_4$ production. Apparently the presence of sludge components and, still more so, of methane bacteria interferes with the separation of the oil from the clay with pentane.

A different behaviour was found in most of the wax cultures as is borne out by the following figures:

| Culture | g of wax lost | hydrocarbon equivalent |
|---|---|---|
| J 7 | 14.5 | 11.2 |
| J 9 | 11.2 | 5.0 |
| J 10 | 1.6 | 0.7 |
| J 13¹) | 13.4 | 12.2 |
| J 14¹) | 0.0 | 0.0 |
| K 14 | 16.2 | 14.2 |

As the amount of recovered oil was usually 30–40 g, analysis according to current methods for crude oils was no easy matter.

At the Amsterdam Laboratory the fractions 180, 180-250, 250–

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¹) From these cultures the wax was recovered at the Royal Dutch-Shell Laboratory at Amsterdam using acetone instead of pentane. The clay-wax-water mixture was first dehydrated with acetone and later on extracted with the same solvent.

325, 325–400 and > 400°C. were obtained using a micro-fractionation method. The ratios of these fractions did not show significant differences between cultures with and without development of a methane fermentation, but the wax content was distinctly lower in oils from cultures with a good fermentation than in oils from those in which no fermentation had occurred. A few examples of total wax contents of crudes are given below.

| Culture | Age, months | % wax | Mpt. °C. |
|---|---|---|---|
| original H P O I | — | 17.5 | — |
| $\varphi$ c oil blank | 55 | 16.1 | 51.5 |
| F 2 | 6 | 19.0 | — |
| F 5 | 12 | 10.7 | — |
| F 3 | 24 | 7.3 | — |
| F 6 | 30 | 8.4 | — |
| F 1 | 44 | 3.8 | 48.5 |
| $\varphi$ c | 55 | 4.0 | 50.0 |
| $\eta$ 1 c | 56 | 3.5 | 54.8 |
| K 10 [1]) | 27 | 75.1 | 44.3 |
| K 9 [1]) | 27 | 46.2 | 40.5 |

The figures for the wax contents of the recovered oils may well deviate ca. 2% from the true values [2]), but nevertheless they clearly demonstrate that in those cultures where an appreciable methane fermentation had developed a large part of the crystallizable alkanes had been converted.

A change of the recovered oil as compared with the original one was already manifested by its appearance: the original H P O I and that recovered from cultures without fermentation was solid at room temperature with a dull surface, that from F 2 was still solid, but had a shiny surface, whereas the oil from other well-developed H P O I cultures was a homogeneous liquid at room temperature.

## § 5. DISCUSSION.

The investigation has shown that bacteria exist which can convert higher alkanes to methane and carbon dioxide. As the results with some of the wax cultures show, this conversion is probably complete, i.e. when a hydrocarbon molecule is attacked, $CH_4$ and $CO_2$ are the only products resulting from this attack [1]).

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[1]) K 9 is the E H P O I culture and K 10 the corresponding oil blank.

[2]) In K 9 and K 10 the deviation will probably be larger than about 2%.

These bacteria are also capable of attacking these hydrocarbons when they are part of certain types of crude oil, especiably heavy paraffinous crudes. However, the conversion did not occur when the crudes contained a notable amount of aromatic gasoline hydrocarbons. The failure of the bacteria to attack hexadecane to an appreciable extent shows that probably the hydrocarbon chain must have a minimum length of more than 16 C atoms in order to be susceptible to attack. This is in line with the experience that crude oils which did not contain crystallizable paraffin wax did not yield any methane, although these oils certainly contained lower alkanes.

The remarkable maximum occurring in the rate of methane production from a heavy paraffinous crude in the course of time may be explained as follows. In these crudes part of the wax crystallizes at temperatures at least up to 30°C. (the temperature at which the cultures were kept) in such a way that the droplets of oil dispersed in the clay-water mixture are irregular in shape so that the wax crystals "stick out through the surface of the droplets".

It stands to reason that the bacteria (which only develop at the oil/water interface) will start to attack the latter part of wax crystals and that, as soon as the droplets have been "rounded off", the rate of attack will decrease and, thus, the methane production as well. In the oldest cultures $\eta$1c and $\varphi$ c methane production had practically stopped when they were broken off, while the analysis of the recovered oils shows that a small amount of crystallizable matter was still left. Presumably diffusion of the last part of the crystallizable alkanes to the oil/water interface proceeds so slowly that the bacteria cannot keep up a measurable fermentation rate.

The fact that a similar maximum occurred in cultures containing small particles of crystallized wax instead of oil droplets may be explained by assuming that the bacterial attack will prefer the edges of the wax crystals to their sides which will also lead to a certain "rounding off" of the crystals. The fact that the level to which the fermentation rate drops after the maximum is much higher in the wax than in the oil cultures is in line with this idea.

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[1]) Of course some assimilation of substrate to cell material will have occurred. However, in anaerobic processes the percentage of substrate assimilated is quite small (e.g. 5%).

The average $CH_4/CO_2$ ratio in the total gas production of the well-developed cultures is somewhat higher than the theoretical one of 3.0 for a complete conversion of higher alkanes to $CH_4$ and $CO_2$.

This relatively small difference may be explained by assuming a certain $CO_2$ retention by the clay or other constituents of the medium.

## Summary.

1. Certain methane bacteria appeared to be able to convert part of the higher alkanes in some heavy paraffinous crude oils to methane and carbon dioxide according to the equation:

$$4\ (CH_2) + 2\ H_2O = 3\ CH_4 + CO_2.$$

2. Bacteria with this ability were found in the sludge from a waste liquor settling pond of a strawboard mill.
3. No methane fermentation occurred with a light paraffinous crude unless it had been topped to 200°C. This is ascribed to a toxic action of the aromatics in the gasoline fraction.
4. A more vigorous fermentation occurred with paraffin wax, leading to a notable conversion of the wax to $CH_4$ and $CO_2$ in the course of a few years.
5. Iso-alkanes from wax were fermented more slowly than n-alkanes, while pure hexadecane was not fermented.

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# *STREPTOSPORANGIUM* ISOLATED FROM FOREST LITTER IN THE NETHERLANDS[1]

by

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(Received August 20, 1957).

In 1949 Couch published a paper on a species of a new group of the *Actinomycetales* which he described in 1950 as *Actinoplanes* (1). The new family was described in 1955 by the same author (2) as *Actinosporangiaceae* and a second genus was introduced, *Streptosporangium*. In 1955 he proposed (3) to change the name *Actinosporangiaceae* to *Actinoplanaceae* for nomenclatural reasons. The spores of the *Actinoplanaceae* are formed in sporangia. The spores of *Actinoplanes* are motile, those of *Streptosporangium* non-motile. *Streptosporangium* is very similar to *Streptomyces*, only it forms sporangia.

The *Actinoplanes* described by Couch was found in soil from the Philippines, *Streptosporangium* in dung and gardensoil in the United States. After Couch had isolated these new forms, only Gaertner (4) has found *Actinoplanes* again. He isolated it from African soils and from marshland soils (Marschböden) in Northwest Germany.

In 1954 the second author isolated cellulose-attacking microorganisms from litter in an oak forest planted on heavy clay soil, poor in inorganic nutrients and with a pH 4.1. The litter is broken down in one to two years. During part of the winter months the water level may reach the level of the soil surface.

The cellulose attacking microorganisms were fungi, actinomycetes, bacteria and myxobacteria (*Cytophaga*). Among the cellulose attacking *Actinomycetes* as *Streptomyces* and *Micromonospora*, the newly described *Streptosporangium* could be found.

*Streptosporangium* was isolated from the upper as well as from the lower layer of the litter. The litter was shaken with water and the *Streptosporangium* was isolated from the dilutions of 1–1 up to 1–10.000 in the months of March, August, September and November. This shows that it is an organism which appears regularly in this oak forest litter.

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[1] Tevens verschenen als Mededeling Nr. 36 I.T.B.O.N.

From forest litter of an oak forest on sandy loam soil *Streptosporangium* was isolated from three different places with a pH varying from 4.6–5.0 and every time from a dilution of 1–1000. In the same forest no *Streptosporangium* has been isolated yet from an area with pH 7.1.

In Denmark *Streptosporangium* was found in Geels Skov near Lyngby on litter under beech trees and Douglas with respectively a pH 5.7 and 4.3.

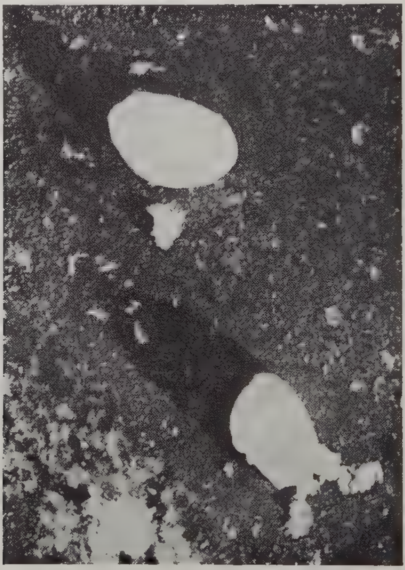


Photo 1.
Electron photo of spores. 18.700 ×. (shadow cast : Palladium).

When the 17 isolates of *Streptosporangium* were grown on oatmeal agar they could be divided into 6 different colour groups, according to the colour card of RIDGWAY (5). Four were "wax yellow", four "pale purplish gray" to "deep slaty brown", five "Vandijke red" to "Hay's maroon", two "carrot red", one "pale pinkish buff" and one "apricot buff" to "wood brown". The aerial mycelium

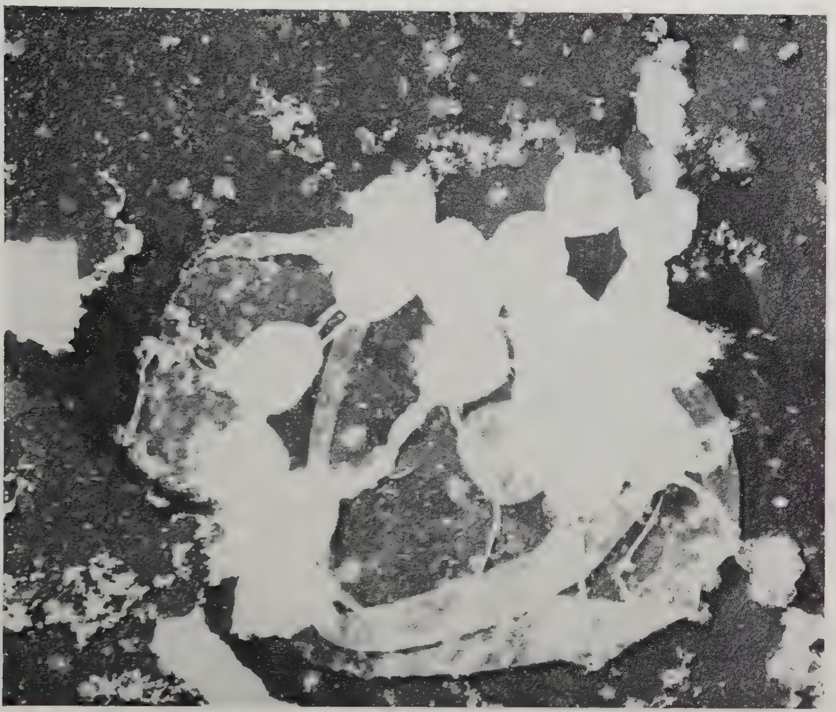


Photo 2.

Electron photo of sporangium. The sporangium wall can be seen as a thin membrane. The spores appear in strings. 18.700 ×. (shadow cast : Palladium)

was powdery white. As with *Streptomyces*, the spores were found in this aerial mycelium. In all the isolates the sporangia had the form of a sphere. Often the aerial mycelium formed a matrix in which the sporangia could be found.

The four isolates which are pale purplish gray on oatmeal agar form purplish crystals in the medium.

The first author studied the development of some of these isolates. An isolate which on oatmeal agar was wax yellow, was studied in detail. The development was followed in a hanging drop culture on Czapek's agar with addition of yeast extract or on agar from leaf extracts of the grass *Hordeum murinum*.

The spores are 1.2–2 $\mu$ long and 0.8–1.0 $\mu$ wide and have a rather thick but not very strong wall. It is difficult to moisten these spores. Under the electron microscope the spores are oval (photo 1), often with flattened facets. The spores are non-motile and have no flagella. Artefacts caused by staining might be mistaken for flagella. Under the electron microscope however no flagella can be found (photo 2).

When the spores germinate, one or two (seldom three) germination tubes develop (figs. 1, 2, 3, 6 and 7). The fine mycelium grows radially and does not penetrate deep into the agar (figs. 4, 5, 7a and 8). In liquid it grows as globular masses. The hyphae are unsegmented.

After the initial growth rate of the mycelium declines, aerial mycelium develops in concentric zones. The length and ramification of this aerial mycelium is different for the various strains. On Czapek's agar its development sets in after 10 days. Its hyphae are sturdier and have thicker walls than the mycelium growing in the substrate. When this mycelium has developed it takes one to two weeks before the formation of the sporangia starts. On grass medium there is no such delay. The tips of the aerial mycelium start to swell and the swollen ends develop into globules which may reach a size of 5–8 $\mu$ (figs. 9–14).

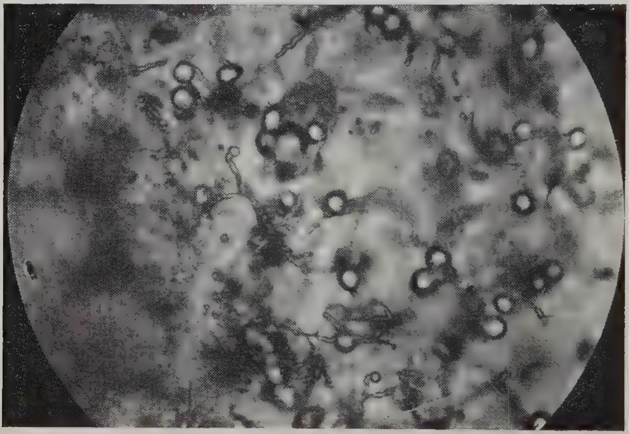
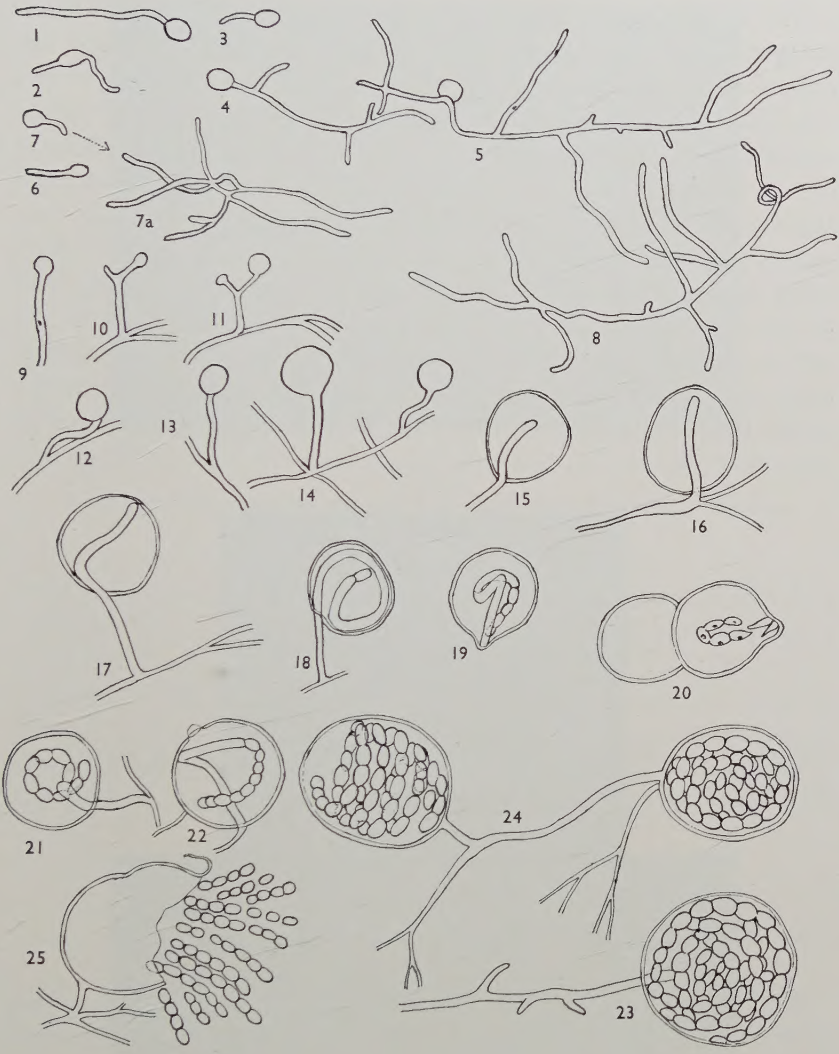


Photo 3.
Development of globules, stage before sporangia develop. 400 ×.

Again on grass leaves the development of these globules to sporangia sets in immediately, on agar it may take some weeks. The globules are optically empty and have a rather thick wall. The stalk of these globules is variable in length and more or less branched.



Figs. 1–25. *Streptosporangium* strains, on Czapek agar with 0.02% yeast extract. All 1500 ×.

Figs. 1–8. Germinating spores and young vegetative mycelia.

Figs. 9–14. Formation of globules at the tips of aerial mycelium.

Figs. 15–17. Penetration of the aerial hypha into the globule.

Fig. 18–22. Formation of spores at the free end of the penetrating hyphe.

Figs. 23 and 24. Sporangia with strings of spores.

Fig. 25. Unripe sporangium with spores pressed out.

Figs. 1 and 2. isolate 391a. Figs. 3–5. isolate 389a. Figs. 6–8. isolate 539 c. Figs. 9–25. isolate 569a

The aerial hypha on which this globule has developed starts to grow into this globule with no change in diameter until the opposite side of the globule is reached (photo 4, figs. 15, 16 and 17). This thread follows the curvature of the globule (photo 5 and 6) and in this way a spiral develops (photo 7 and fig. 18). After some curves of the spiral the free end of the hypha forms septa and the spores are formed (figs. 19–22). During this period the sporangium can grow to a size of 8–18 $\mu$. The spores remain in a spiral until the sporangium has ripened (figs. 23 and 24). Now the wall of the sporangium becomes thinner until it is invisible with the ordinary microscope, but is still apparent under the electron microscope as a thin membrane. If the sporangium is opened in a unripe stage the spores appear in chains (fig. 25). At this stage the electron microscope shows that there are still connections between the spores.

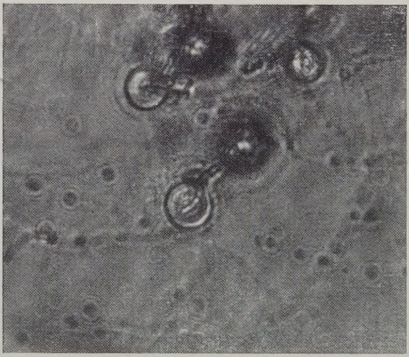


Photo 4.
Growth of the hypha into the globules. 1000 ×.

Couch (1955) has already described the formation of the spores in spirals in the sporangium and the manner in which septation of the hypha into spores takes place. *Streptosporangium roseum* the type species described by Couch, forms next to the sporangia also threads wound in spirals without the existence of a sporangium. They develop into spores, exactly as in *Streptomyces*. In the isolates described in this paper only the spore formation within sporangia could be found.

All these *Streptosporangium* isolates were found on cellulose agar. The cellulose was prepared according to the method of Scales (6) and the agar contained $K_2HPO_4$, $NH_4NO_3$, $MgSO_4$ and soil extract.

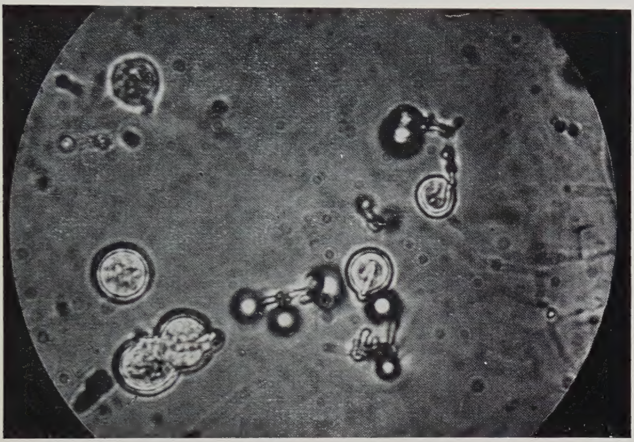


Photo 5.
Sporangia with curved threads and with threads forming regular constrictions. 1000 ×.

Only organisms attacking cellulose were isolated. Some of the pure cultures did not attack cellulose until an extract of rabbit dung or yeast autolysate was added.

A good growth was obtained on saccharose, maltose, lactose, glucose and glycerol. There was poor growth on d-arabinose and no growth at all on d- and l-xylose. Addition of yeast extract improved the growth. Good growth was accompanied by the formation of acid.

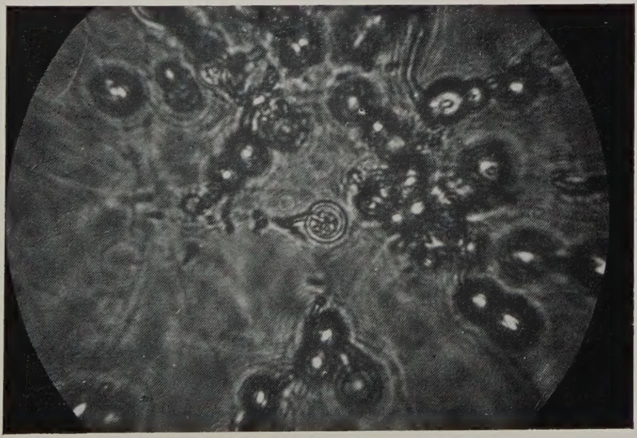


Photo 6.
Sporangium with curved thread. On the free tip septa are formed. 750×.

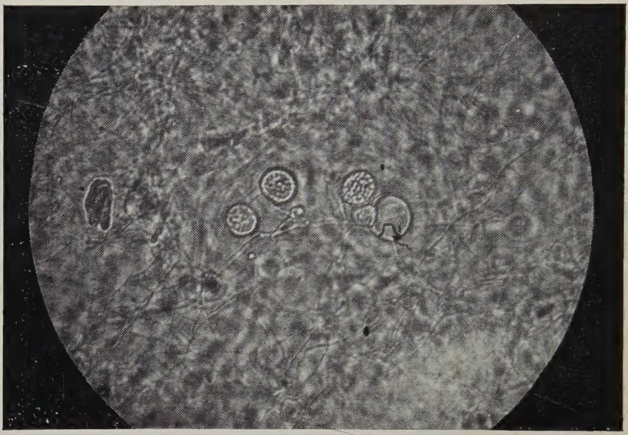


Photo 7.
Sporangia with strings of spores. 750 ×.

Whether or not nitrate was included in the Czapek agar made no difference in the growth. This was also the case with aspartic acid, valine and isoleucine. With alanine and with leucine the growth was more or less reduced, while with glutamic acid no growth at all occurred. In the last case the change in pH after sterilisation probably caused the growth suppression.

## Summary.

The organism described by Couch as *Streptosporangium* was isolated from forest litter in the Netherlands and Denmark. The development of this organism is followed in detail.

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